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EFFECT OF SUBOPTIMAL TEMPERATURE ON THE GROWTH AND VIABILITY OF *SALMONELLA*

by

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ABSTRACT

The effect of suboptimal temperature on growth and viability of salmonellae in tryptic soy broth (TSB) and glucose-salt broth (GSB) at pH 5, 6 and 7 was studied using a static temperature gradient incubator. Survival or growth of *Salmonella heidelberg*, *Salmonella typhimurium* and *Salmonella newport* in TSB at all pH levels was essentially the same from 0-30°. Minimum growth temperatures were approximately 14, 6 and 6° at pH 5, 6 and 7 respectively. Total growth as measured by viable count and absorbance was highest at pH 7. Growth of *S. heidelberg* in GSB was slower and much less extensive compared with TSB. The organism could grow in GSB at 11, 9 and 9° at pH 5, 6 and 7 respectively. Survival was favored at lower temperatures of incubation.

Subsequent experiments were carried out using *S. heidelberg* incubated in GSB under aerated conditions. Between 37 and 18°, growth rate was a linear function of temperature. The linearity was distorted at temperatures below 18° where growth rate was greatly retarded. Similarly the yield coefficient was relatively constant from 37 to 18° but below this range yield was no longer independent of growth rate and fell off precipitously. The defect in low temperature growth at 7, 9 and 15° was also evidenced by lag periods of 12, 8 and 2 hr respectively. During the lag period, ATP synthesis dropped rapidly but was resumed just before the initiation of growth. Appreciable amounts of ATP were synthesized even at 7 and 9°. The aerated culture showed growth at 6-7° in GSB compared to 9° in the static temperature gradient incubator.

Growth of *S. heidelberg* did not occur at temperatures below 6° either in TSB or GSB. Instead the organism showed metabolic injury as

manifested by an increased sensitivity to selective media (desoxycholate agar and MacConkey agar). The rapidity and extent of injury increased with decreasing temperature from the minimal growth temperature to 0°. Leakage of U.V.-absorbing compounds from the organism occurred during cold storage and the cells became permeable to 8-anilino-1-naphthalene sulphonic acid. Degradation of RNA, DNA and protein was not significant during cold storage. Addition of metabolic inhibitors to the storage menstrua did not change the percentage of injury significantly, with the exception of 2,4-dinitrophenol which appeared to exert a protective effect. On transfer to 20°, the culture exhibited an extended lag period during which it rapidly recovered its tolerance to selective media. Recovery was favored at pH 6-8 and 25-35° but could not be demonstrated at pH 4 and 10 or at 5°. The presence of metabolic inhibitors in the recovery menstrua did not prevent recovery. Practically no increase in levels of RNA, DNA and protein was found during the recovery period. Recovery could occur in simple substrates such as phosphate buffer and distilled water.

Another phenomenon was observed immediately upon transferring an exponential culture in GSB from 20 or 37° into fresh medium pretempered at 5°. The culture was rendered unable to grow on tryptic soy agar + 0.5% yeast extract whereas growth on glucose-salt agar was abundant. On further incubation at 5°, the culture gradually recovered from such a sensitivity within 8 hr. Recovery depended on RNA synthesis and was much faster at 20°. However, the culture grown in TSB did not show such a 'minimal medium recovery' effect.

The extent of injury and recovery was also markedly affected by the storage menstruum. Cultures grown in GSB and stored in distilled water

at 2° were also rendered sensitive to desoxycholate agar, but the recovery process required ATP and mucopeptide synthesis. The stored cells lost their viability on tryptic soy agar + 0.5% yeast extract rapidly when incubated at 25° in distilled water but recovered cells survived under the same conditions.

It is concluded that cold injury and recovery are very complex phenomena, markedly affected by experimental conditions. In particular the results emphasize the desirability of non-selective pre-enrichment techniques and the need for re-examination and evaluation of selective plating media for the isolation and detection of salmonellae from foods held at refrigerated temperatures.

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INTRODUCTION

In food preservation and processing, temperature plays a vital role. Heat sterilization, pasteurization, freeze drying, freezing and low temperature storage are in widespread use. It is, therefore, important for the food microbiologist to gain an understanding of the physiological or biochemical factors that determine the temperature limits for microbial growth, metabolic activities at temperatures other than optimal growth temperature, and survival and damage of micro-organisms at non-permissive growth temperatures.

A useful way of expressing quantitatively the effect of temperature on a microbial activity is in the form of the temperature co-efficient or Q_{10} value, which is the ratio of the rate of a metabolic process at one temperature to the rate at a temperature 10° lower. Another more sophisticated method is by use of the Arrhenius equation,

$$V = A_e^{-E/RT}$$

where V = reaction velocity

A = entropy constant

E = activation energy

R = gas constant

T = absolute temperature

A plot of the natural logarithm of V against the reciprocal of the absolute temperature T results in a linear relationship. Arrhenius later modified the equation when dealing with biological systems by replacing E with μ, which is referred to as the "temperature characteristic". However, unlike chemical reactions, the linear relationship

between biological processes including microbial growth and enzymatic reactions and temperature only holds over a narrow range of temperatures. This is because in cellular systems a whole sequence of enzymatic reactions is involved, all of which have their individual temperature coefficients and heat-stability, therefore a greater degree of complexity is only to be expected.

Microbial growth is found to be best in a rather restricted range of temperatures falling off correspondingly at temperatures above and below this range. At the extreme, growth is not possible and these two limits are referred to as the minimum and maximum growth temperatures. The temperature or narrow range of temperatures at which the organism grows best is known as the optimum temperature. In a living system, the optimum temperature may be termed as that point at which metabolic processes function at a maximum rate consonant with the maintenance of the system. In bacteriology, the most common meaning of optimum temperature is the temperature at which the specific growth rate is maximal (Ingraham, 1962).

The fact that all micro-organisms exhibit a minimum, optimum and maximum growth temperature and that these temperature criteria vary between organisms has been used to separate micro-organisms into three main groups, namely psychrophiles, mesophiles and thermophiles. Psychrophiles are a rather ill-defined group of micro-organisms, distinguished from the others by their ability to grow at low temperatures and now generally accepted as micro-organisms capable of producing microscopically visible colonies on a solid medium within two weeks at 0° (Ingraham and Stokes, 1959). However, Stokes (1963) later restricted this definition and confined the required formation of

visible colonies to one week. Farrell and Rose (1967a), further divided the psychrophiles into obligate and facultative psychrophiles according to their optimum growth temperatures. Psychrophiles with an optimum growth temperature of greater than 20° were termed facultative psychrophiles while those with an optimum temperature of less than 20° were termed obligate psychrophiles.

Thermophilic micro-organisms are defined as micro-organisms having a maximum growth temperature above 50° (Farrell and Rose, 1967a). Thermophiles can be further subdivided into obligate and facultative thermophiles. An obligate thermophile has an optimum growth temperature between 65 - 70° and a minimum of 40 - 42° while a facultative thermophile has a maximum growth temperature between 50 - 65° and a minimum of room temperature or less (Farrell and Campbell, 1969).

Mesophiles are generally characterized by their growth optima which lie between 25° and 40°. In general, the most favorable temperature for growth of the organism is roughly correlated with the natural habitat, thus most, if not all the bacteria that are pathogenic to or saprophytic on man are mesophiles with a growth optimum around 37°. Elliot and Heiniger (1965) used a temperature gradient incubator to study the growth of 14 strains of *Salmonella* in trypticase soy broth at super-optimal growth temperatures. They found that the maximal growth temperatures fell between 43.2 and 46.7° and that no strain at 10^6/ml survived 50° for 48 hr. On the other hand, Matches and Liston (1968) found the minimum temperatures, as determined by visible growth on agar for 7 serotypes of *Salmonella*, ranged from 5.5 to 6.8°, whereas in broth culture, after 19 days' incubation, the minimum growth temperatures for *S. heidelberg*, *S. typhimurium* and *S. derby* were 5.3, 6.2 and 6.9°

respectively.

I. Microbial Activities at Superoptimal Temperatures.

From the above information, it might be asked what is the physiological or biochemical basis for maximum growth temperature of a micro-organism? Some plausible suggestions of factors limiting microbial growth above maximum growth temperature include the denaturation of respiratory enzymes (Edwards and Rettger, 1937; Hagen and Rose, 1961, 1962; Evison and Rose, 1965); changes in the properties of membrane lipids (Luzzati and Husson, 1962; Byrne and Chapman, 1964) and membrane protein (Inouye and Pardee, 1970; Siccardi *et al.*, 1971); denaturation and degradation of RNA (Califano, 1952; Strange and Shon, 1964; Pace and Campbell, 1967; Allwood and Russell, 1968); the selective leakage of cellular components especially RNA derivatives as a result of rupture of the cell membrane (Strange and Shon, 1964; Hagen *et al.*, 1964; Haight and Morita, 1966; Malcolm, 1967). The work done in this department has shown that temperatures of 30° have no deleterious effect on the activity of all enzymes of the Krebs' cycle in the psychrophile, *Micrococcus cryophilus* (Malcolm, 1967). The most important role in determining the heat sensitivity of this organism was played by enzymes associated with RNA synthesis and/or degradation (Tai, 1967). The results of Lee (1968) and Gray (1969) showed a decrease in RNA content of *M. cryophilus* at temperatures a few degrees above the maximum growth temperature. Malcolm (1969) reported that both prolyl and glutamyl-tRNA synthetases are temperature sensitive and further suggested these two amino acid activating enzymes to be the molecular determinants of the

maximum growth temperature of *M. cryophilus*, with RNA degradation being a secondary factor. Later Gray *et al.* (1973) found that degradation of the 23S and 16S RNA species of *Pseudomonas fluorescens* occurred at 36°, a temperature just above its growth maximum and the partially destroyed RNA species could be resynthesized at the optimal temperature of the organism. Campbell and Pace (1968) examined the differential heat stability of ribosomes from a variety of psychrophilic, mesophilic and thermophilic micro-organisms and found the thermal stability of ribosomes correlated positively with the maximal growth temperature of the organisms.

At temperatures above the optimum, the growth rate of micro-organisms usually declines very rapidly. Forrest (1967) found the fall in growth rate and yield of *Zymomonas mobilis* began at a temperature a little above the accepted optimal temperature of the organism and was not accompanied by a similar decrease in glycolytic activity and the rate of degradation of glucose continued to increase at high temperatures. He suggested that the maximal efficiency of growth might be realized only over a restricted range of temperature and that outside this range energetically uncoupled growth could take place even under conditions of adequate nutrition. Ron and his colleagues (1971a,b) suggested the immediate reversible heat-sensitivity of homoserine transsuccinylase of *Escherichia coli* was responsible for its decreased growth rate at 45° compared to 40°.

II. Microbial Activities at Suboptimal Temperatures.

A. Physiological Activities

Pigment production: Synthesis of the red pigment, prodigiosin, by strains of *Serratia marcescens* is favoured between 20 and 25°, although the optimum temperature for growth of the bacterium is near 37°. The enzyme that catalyses the final step in the biosynthesis of prodigiosin, the coupling of a monopyrrole with a bipyrrole to give the linear tripyrrole, prodigiosin, is abnormally temperature-sensitive (Williams *et al.*, 1965). On the other hand, the production of a red pigment by the silkworm pathogen, *Bacillus cereus* var. *alesti* at 15° but not at 25° has been traced to the effect of temperature on enzyme synthesis rather than on enzyme activity.

Flagella production: Production of flagella is often favoured at low temperatures and is absent at higher temperatures; such examples include *Bacillus inconstans* (Braun and Löwenstein, 1923), *E. coli* (Morrison and McCapra, 1961), *Salmonella paratyphi B* (Jordan *et al.*, 1934), *Salmonella typhi* (Felix *et al.*, 1934) and certain psychrophilic bacteria (Schubert and Schubert, 1953). Roberts and Doetsch (1966) studied the effect of temperature on regeneration of flagella in bacteria that had been experimentally deflagellated, and showed that the thermophile, *B. stearothermophilus* 11330 resynthesized flagella at 20°, a temperature below the minimum for the growth of the bacterium.

Polysaccharide synthesis: There is a tendency for micro-organisms to synthesize increased amounts of polysaccharides at suboptimal temperatures. Production of extracellular dextrans by leuconostocs and pediococci is well known to be favoured at temperatures below the optima

for growth of these bacteria. This effect has been attributed to the production by these bacteria of a dextransucrase that is very rapidly inactivated at temperatures >30° (Neely, 1960) and, with a lactobacillus, to the temperature-sensitive nature of the dextransucrase synthesizing system (Dunican and Seeley, 1963). Tempest and Hunter (1965) reported an increase in the content of cell carbohydrate when the temperature of *Aerobacter aerogenes* in continuous culture was decreased from 35 to 25°. Ng (1969) found that *E. coli* grown at 10° are richer in carbohydrate (22% of dry weight) than are cells grown at 37° (8.4% of dry weight).

Sugar fermentation: Greene and Jezeski (1954) found that sugar fermentation at temperatures below 30° gave rise to both acid and gas, while above 30° only acid was produced. Similarly, Upadhyay and Stokes (1963a,b) studied a psychrophile which fermented glucose and other sugars with the formation of acid and gas at 20° and lower, but produced only acid at higher temperatures. This difference was ascribed to a temperature-sensitive hydrogenase synthesizing system of the cell. Beef spoilage bacteria have been shown to liquefy gelatin and utilize water-soluble beef proteins more at 5° than at 30° (Jay, 1967), but whether this effect is due to temperature-sensitive enzymes is not yet known.

B. Molecular Basis for the Minimum Temperature for Growth

The physiological basis for the fundamental difference between psychrophiles and mesophiles, namely the ability to grow at 0°, has interested several groups of workers over the past decade. As yet, however, no firm understanding of this ability has been reached.

Several theories have been put forward to explain the relatively high minimum temperature for growth of mesophiles. The first of these was formulated as a result of observations of Ingraham and Bailey (1959)

and later of Baxter and Gibbons (1962) and Rose and Evison (1965). It postulates that mesophiles are unable to grow at temperatures below 5-10° because, at these temperatures, they are unable to transport solutes across the cytoplasmic membrane. Farrell and Rose (1967b) have offered three basic mechanisms by which low temperature could affect solute uptake: (a) inactivation of individual permease-proteins at low temperature as a result of low temperature induced conformational changes which have been shown to occur in some proteins, (b) changes in the molecular architecture of the cytoplasmic membrane which prevent permease action, and (c) a shortage of energy required for the active transport of solutes. According to Farrell and Rose, the possibility of (a) does not seem likely, mainly because this would mean that the minimum temperature of a micro-organism would vary with the chemical composition of the medium, an observation which has not been reported. The possibility of (c) is also unlikely, because endogenous respiration proceeds at temperatures below the minimum for growth (Baxter and Gibbons, 1962; Rose and Evison, 1965); moreover, the ATP contents of *Candida utilis* grown at 25 or 10° are almost identical. This leaves (b) and it has been suggested that, at near-zero temperatures, the fatty acid side chains in membrane phospholipids of the mesophile solidify (Byrne and Chapman, 1964) and this could restrict the mobility of the permease proteins. However, Marr and Ingraham (1962) found that growth at a particular temperature does not result in a unique fatty acid composition, since altering the nutrition status independently of temperature also resulted in major changes in fatty acid composition. They concluded that the fatty acid composition of the bacteria does not determine the minimum temperature for growth.

Inactivation of solute transport in mesophiles at low temperatures was discovered as a result of preliminary observations by Ingraham and his colleagues. However, more recent work in Ingraham's laboratory has been concerned with another aspect of the physiology of micro-organisms at low temperatures. Ingraham and Maaløe (1967) believe that, at temperatures below the optimum for growth of mesophilic micro-organisms, there is a progressively more effective derangement of the biochemical mechanisms that regulate the activity and synthesis of enzymes and that this derangement leads ultimately to cessation of growth. Long before a molecular basis for metabolic regulatory processes had been proposed by Jacob and Monod (1961) there had been reports that, in general, induction of enzyme synthesis by micro-organisms was very temperature sensitive (Knox, 1953). More recently, Halpern (1961) reported that synthesis of glutamate decarboxylase in *E. coli* is inducible at 37° but partly constitutive at 30°. In addition, tryptophan has been shown to induce tryptophanase synthesis in another wild strain of *E. coli* at 30° but not at temperatures <15° (Ng and Gartner, 1963). Repression of enzyme synthesis is also often sensitive to temperature. Gallant and Stapleton (1963) isolated a mutant of *E. coli* B in which the extent of repression of alkaline phosphatase synthesis by inorganic phosphate decreases as the temperature is increased from 20 to 40°. Marr *et al.* (1964) showed that the maximum differential rate of β-galactosidase synthesis in *E. coli* ML30 in either a constitutive strain or a maximally induced wild-type strain is significantly reduced if the culture is grown at low temperature. A report by Ng *et al.* (1962) suggested that the growth of *E. coli* ML30 at low temperatures damaged the bacteria in such a way that the growth rate was decreased.

These workers showed that the glucose repression of β-galactosidase synthesis by this organism occurred at a temperature that coincided with damage to the bacterium. It has been suggested on the basis of these results that the temperature affects the synthesis of repressor proteins (Marr *et al.*, 1964), that the affinity of the repressor for the co-repressor is influenced by temperature (Farrell and Rose, 1967a), or that the repressor molecule itself is thermolabile (Udaka and Horiuchi, 1965). As yet, however, no one has demonstrated that these effects can contribute to the high minimum temperature for growth of mesophiles.

Ng *et al.* (1962) studied the effect of shifting an exponentially growing culture of *E. coli* ML30 from 30° to 10° and found that there were no changes in turbidity and viable counts for 4 hr. It was later found by Shaw and Ingraham (1967) that during this lag period, there was no net synthesis of deoxyribonucleic acid, ribonucleic acid and protein. In view of their results that protein synthesis commenced after 4 hr of lag and that growth did not recommence till after four and a half hours, it would appear that protein synthesis was necessary for the resumption of growth at low temperature.

According to Kaempfer *et al.* (1968), initiation of protein synthesis in *E. coli* involves the ordered association of the two ribosomal subunits, 50S and 30S, formylmethionyl-transfer RNA (F-met-tRNA) and several protein factors on the initiation signal of the mRNA. After the 70S ribosome completes the translation of the messenger RNA molecule, it dissociates at some point into subunits before re-initiating protein synthesis once again. It has been shown (Das and Goldstein, 1968) that at 0° protein synthesis by *E. coli* slows progressively and

eventually stops. However, it is restored instantly to normal if the cells are warmed to 37°. The cause of the low-temperature defect appears to be an inability of free ribosomes to attach to messenger RNA. Their evidence suggests that a ribosome has to be activated especially, once it has been released from the 3' end of a messenger RNA strand, in order to permit its functional reattachment at the 5'-end of a new operon. This activation apparently proceeds too slowly at 0° to be effective in competition with inactivation processes. They suggested the low temperature effect on protein synthesis would explain the well-known phenomenon of minimum growth temperature. Friedman *et al.* (1969) also found that when an exponentially growing culture of *E. coli* is cooled to below 8°, there is an immediate accumulation of 30S and 50S ribosomal subunits due to a block in the initiation of protein synthesis while the elongation of initiated proteins continues until they are completed. This low-temperature ribosomal subunit accumulation has a sharp cut-off between 8° and 10°. At 10°, no accumulation of subunits occurs. Most other activities including RNA and DNA synthesis continue at 7°. They suggest the failure to initiate the synthesis of new proteins at sub-minimal growth temperature is responsible for failure to carry out cell division, a process which naturally requires new proteins.

It is still not known to what extent uncoupling of energy production is the basis for the minimum temperature for growth. Forrest (1967) reported that growing cultures of *Streptococcus faecalis* at temperatures from 30 to 36.7° had activation energies for both rates of growth and glycolysis of 10.3 K_{cal} $mole^{-1}$, and a constant growth yield, when growth took place below this temperature, the growth yield

decreased and the activation energy for growth increased to 21.1 K_{cal} $mole^{-1}$, but the activation energy for glycolysis was unchanged. The adenosine triphosphate pool in the organism behaved differently above and below 30°, suggesting that the energetic coupling between anabolism and catabolism is less effective below 30°. Ng (1969) had similar findings with *E. coli* ML30, the yield of which began to fall off precipitously at temperatures below 15°. His observation that growth was most rapid on glucose, slowest on succinate and intermediate on glycerol at 37°, whereas at 13° the rate of growth was equal on all three carbon sources, suggests that catabolism of the carbon source is the limiting factor of growth at higher temperatures and biosynthesis limits growth at low temperatures. There was no defect in energy production at temperatures down to 10° and the energy produced was actually stored in the form of glycogen since the glycogen to protein ratio of cells grown at 10° was approximately three times higher than that of cells grown at 37°. Ng (1969) suggested that biosynthesis limits growth at low temperatures and that decrease in cell yield with decrease in temperature is a result of uncoupling of energy production from energy utilization and not due to decreased permeability at low temperatures.

The available evidence cited above indicates that the block in initiating protein synthesis at low temperatures is most likely the molecular basis for the minimal temperature for growth of a mesophile, although further experimentation is clearly needed.

III. Sublethal Temperature Effect on Micro-organisms.

When bacteria are exposed to conditions such as heat, freezing, drying, irradiation and chemical treatments, both death and injury may occur. Injury represents the loss of some characteristic of the untreated cells, which restricts growth after treatment, if this specific characteristic is challenged. Injured cells are generally able to repair this damage under favorable conditions and hence recover their ability to grow as well as the untreated cells. Sublethally-injured micro-organisms are frequently encountered in food products whether partially heat-processed, frozen, dried or acidified. The most frequent manifestation of injury is the increased sensitivity of damaged cells to selective media. This observation has important implications with regard to the detection and enumeration of such organisms.

A. Sublethal Heating

These conditions of injury and recovery are well illustrated by *Staphylococcus aureus* when sublethally-heated cells exhibit an increased sensitivity to salt and are able to regain their salt tolerance when exposed to suitable conditions.

Expressions of injury that have been reported include: extension of the lag phase of growth; more exacting nutrient, temperature and pH requirements; reduced oxygen consumption and increased sensitivity to inhibitors and selective agents.

Jackson and Woodbine (1963) showed that when an enterotoxigenic

strain of *Staphylococcus aureus* was subjected to sublethal heat treatment and subsequently inoculated into nutrient broth at 37°, there was a drop in viable count followed by a lag phase of growth. The phenomenon was described as an extended lag phase. More recent work has shown that the extended lag phase is actually a recovery period.

Nelson (1943) in reviewing the literature concerned with recovery of bacteria in various media, considered that 'rich' media gave the highest recoveries, but recent findings indicate that this is not always true. In a later paper Nelson (1956) reported that the pH of the plating medium most favorable for growth of heat-treated organisms differed from that for non-heated cells.

An attempt to determine the factors responsible for the improved growth of heated *Pseudomonas fluorescens* on complex media compared to simple salts-agar medium was made by Heather and Van der Zant (1957). They observed increased survival when casamine acids or a mixture of eighteen amino acids were used to supplement the synthetic medium. Subsequent experiments in which single amino acids were added to synthetic agar supplemented with glutamic acid showed that combining glutamic acid with either lysine, proline or methionine gave counts of heat-treated cells similar to those with all eighteen amino acids present.

The influence of various components of recovery media for heat-treated *E. coli* was studied by Russell and Harries (1968). They found that vitamins were not involved in the recovery process and that although addition of amino acids to a synthetic medium gave increased recovery, addition of yeast extract was even more beneficial.

On the other hand, Gomez *et al.* (1973) found that exponential

phase *Salmonella typhimurium* LT2, *S. thompson* and *S. heidelberg* grown in a glucose-salt medium (M-9), harvested at 37° and heat treated at 50° exhibit a higher recovery on M-9 agar than on trypticase soy agar with yeast extract. This phenomenon is known as minimal medium recovery. Heat-treated bacteria incubated at 37° in M-9 broth or distilled water recovered their ability to grow on trypticase soy agar. In accord with recent reports of thermally induced DNA breakage in *E. coli* (Sedgwick and Bridges, 1972) and its restitution (Woodcock and Grigg, 1972), they presented evidence of the repair of DNA single-strand breakage during recovery (Gomez and Sinskey, 1973).

Harries and Russell (1966) reported increased viable counts of heat-treated *E. coli* when pour-plates were used compared to surface spread-plates. Similar observations were made by Baird-Parker and Davenport (1965) using *Staphylococcus aureus*. They suggested that anaerobic or semi-anaerobic conditions may assist recovery. This finding is interesting in view of the effect of the presence of oxygen during the heating period described above. Allwood and Russell (1966) reported increased counts when either glucose or phosphate was incorporated in nutrient agar used for recovery of heated *Staph. aureus*. In view of the findings of these authors that heated cells grew better at low pH, it may be that acid produced by glucose metabolism accounts for the effect of this compound.

Staphylococcus aureus was found to require a number of substances for recovery after heating (Iandolo and Ordal, 1966). These were given as glucose, a mixture of amino acids and phosphate. Recovery of the cells was found to occur in the absence of cell division. In addition, by incorporation of various inhibitors in the recovery medium, RNA

synthesis was shown to be necessary for the recovery of heated cells. Thus it may well be significant firstly that variations in the stability of RNA occur between organisms having growth optima at different temperatures (Saunders and Campbell, 1966) and secondly that RNA degradation (Eigner *et al.*, 1961) and leakage (Iandolo and Ordal, 1966; Allwood and Russell, 1968) occur on heating bacteria.

Repair to thermal injury of *Staphylococcus aureus* also appears to be involved in the finding of Busta and Jezeski (1963) that heat-induced salt sensitivity could be eliminated by incubation of the damaged cells in a nutrient medium. Subsequently, Sogin and Ordal (1967) showed that ribosomal RNA was resynthesized and Rosenthal and Iandolo (1970) showed that 16S RNA was the prime target of degradation and as a consequence the 30S ribosome subunit was also destroyed during heating while during recovery (Rosenthal *et al.*, 1972) both ribosomal particles were reassembled and the 50S subunit was turned over and used as a source of protein for new ribosome assembly and *de novo* synthesis was limited solely to RNA. Clark and Ordal (1969) investigated the effect of sub-lethal heat upon the growth of *Salmonella typhimurium* on several commercially available selective media. They found injured organisms to be sensitive to brilliant green agar, Levine eosin methylene blue agar, Salmonella-Shigella agar and desoxycholate citrate agar. The organism recovered its ability to grow on these media after incubation in trypticase soy broth. Tomlins and Ordal (1971a) showed that synthesis of ATP, RNA and protein were all essential for the recovery of *S. typhimurium* from injury induced by sublethal heat. They (1971b) further demonstrated that after injury the 16S RNA species was totally degraded and the 23S RNA was partially degraded while the 30S ribosomal

subunits were totally destroyed and the sedimentation coefficient of the 50S particle was decreased to 47S. The rate-limiting step in the recovery of *S. typhimurium* from thermal injury was in the maturation of the newly synthesized rRNA and the synthesis of new ribosomal proteins was also necessary.

Thus present evidence points out strongly the lesion of heat injury of both *Staph. aureus* and *S. typhimurium* results in degradation of ribosome subunits with 16S RNA and 30S ribosome subunits being the main target. Hurst *et al.* (1973) added that heat damaged cells of *Staph. aureus* may recover their salt tolerance while various membrane functions remain impaired. They later (1974) found that there was good correlation between loss of salt tolerance and loss of cellular magnesium and suggested that loss of magnesium was one of the primary events in the sublethal heat injury of *Staph. aureus*.

Nelson (1956) found a pH of 6.0 to be optimal for recovery of heated *Staph. aureus* when incubated at 37° and a similar finding was reported by Allwood and Russell (1966) who also reported the optimal recovery temperature being 32°. It has been stated previously (Iandolo and Ordal, 1966) that a pH of 7.2 was optimal for recovery of this organism when incubated at 37°. However, the experimental techniques of Iandolo and Ordal (1966) differed from those of the other workers.

Alcaligenes species could be recovered from pasteurized milk by maintaining at a temperature of 5° for a period before incubation at 37° (Macauley *et al.*, 1963). Without the period at 5° recovery of viable organisms was not possible. The rate of cooling from the heating temperature to the recovery temperature was stated by Hansen and Rieman

(1963) to affect survival. More organisms survived when the rate of cooling was slow. They proposed that survival may be due to absence of cold shock.

B. Chilling and Freezing

Sudden chilling of a dilute suspension of bacteria from their normal growth temperature to near 0° causes loss of viability (Sherman and Albus, 1923). This phenomenon, which has come to be known as 'cold shock', has since been demonstrated with other strains of *Escherichia coli* and other Gram negative bacteria (Meynell, 1958; Gorrill and McNeil, 1960; Strange and Dark, 1962; Strange and Ness, 1963). Gram positive bacteria are thought to be insensitive to cold shock, although Ring (1965a,b) described a similar phenomenon in *Streptomyces hydrogenans*. Susceptibility to cold shock is usually found only in bacteria from exponential-phase cultures (Gorrill and McNeil, 1960) and is dependent on rapid chilling. The occurrence of cold shock is also affected by the concentration of cells, the medium in which the cells are grown and in which they are suspended during chilling (Gorrill and McNeil, 1960; Houghtby and Liston, 1965; Strange, 1964). Houghtby and Liston (1965) suggested that the resistance of *E. coli* K-12 to cold shock is related to the biosynthetic capability of the cultures since cultures grown in media providing more preformed nutrients are more cold shock sensitive. They also observed that cultures grown at 22° were more resistant to cold shock than cultures grown at 35° and that rapid warming of a culture grown at 22° to 35° prior to cold shock does not render the culture more sensitive. When a thick suspension of *Aerobacter aerogenes* is subjected to cold shock, release of ultraviolet absorbing materials, amino acids and ATP has been observed (Strange and

Dark, 1962; Strange and Ness, 1963; Strange, 1964), suggesting a damage of the cells' permeability barrier. Strange and Dark (1962) reported that sucrose, Mg^{++}, or Ca^{++} as well as bacteria-free filtrates from chilled concentrated suspensions of exponential-phase cultures protected a dilute suspension from the lethal effect of chilling. Recently, Farrell and Rose (1968) have studied the effect of chilling on a mesophilic strain of *Pseudomonas aeruginosa* and a psychrophilic pseudomonad. They found that the psychrophile, which contains a slightly greater proportion of unsaturated lipids, compared with the mesophile when grown at 30°, was less susceptible to cold shock than the mesophile. But when both are grown at 10°, which causes an increased synthesis of unsaturated lipids in the bacterial membranes, they are no longer susceptible. They suggested that cold shock was a result of a sudden release of cell constituents from bacteria, following the 'freezing' of certain membrane lipids after the sudden chilling and the consequent development of 'holes' in the membrane; the insensitivity to cold shock in bacteria grown at 10° could be explained by the lower melting point of membrane lipids and hence no 'holes' were formed in the membrane of these bacteria. Sato and Takahashi (1969) demonstrated that *E. coli*, *P. fluorescens* and *B. subtilis* were susceptible to shock when in the exponential phase of growth and that the viability of cold shocked cells increased rapidly when incubated at 30° when either Mg^{++} or ATP was present in the recovery Tris buffer.

The effects of freezing and thawing upon the microbial cell have been extensively investigated over a period of almost a century. Pictet and Young (1884) exposed *Saccharomyces cerevisiae* to temperatures of -70° for 108 hr and -130° for a further 20 hr and found that the

yeast lost its ability to raise bread. Since then much work has been carried out in an effort to elucidate actual damage to the cell caused by freezing and thawing and to determine the external manifestations of this damage. Populations of coliforms and salmonellae in foods were found to be progressively less able to grow on selective media as compared to non-selective media after storage at sub-zero temperatures (Gunderson and Rose, 1948; Hartsell, 1951). Straka and Stokes (1959) noted that three strains of *Pseudomonas* and one of *E. coli* gave similar colony counts on trypticase soy agar and a minimal glucose-salts agar but after freezing and thawing the counts on the minimal agar were much lower than on trypticase soy agar. Similar results were reported for *Shigella sonnei* (Nakamura and Dawson, 1962) and for *Pseudomonas fluorescens* and *E. coli* (Arpai, 1962).

By this time it had become apparent that any measurement of the survival of cells exposed to freezing was dependent upon the medium used to assay viability. Those bacteria capable of growth on a non-selective and highly nutritious medium but not on a simple minimal salt medium were termed 'metabolically injured'. From this point, efforts were directed towards defining the nutritional modifications induced by freezing and thawing and assessing cellular damage which could influence these alternations. Moss and Speck (1966a) showed that freezing affected the cell membrane as evidenced by leakage of RNA nucleotides and short chain peptides from *E. coli* subjected to freezing. They reported that the peptide fraction offered protection against the effect of freezing when added to the freezing menstruum of a fresh culture prior to freezing. Moss and Speck (1966b) identified short chain peptides in trypticase that were instrumental in promoting growth

of the injured cells, thus confirming the report of Straka and Stokes (1959), that freezing caused certain cells of a bacterial population to lose their ability to use inorganic nitrogen. As a result of studying metabolic injury in *Aerobacter aerogenes* and *E. coli*, Macleod and his colleagues (1966) concluded that freezing damaged the cytoplasmic membranes in the bacteria, with the results that toxic ions, which are present in trace amounts, can penetrate the bacteria. The enriched media permit growth of the metabolically injured bacteria by providing compounds that chelate the toxic metal ions. An extended lag period similar to that caused by sublethal heating has been shown to occur following freezing and thawing (Postgate and Hunter, 1963). Arpai (1963) suggested that the extended lag period was in reality a recovery period during which damage to the cell was repaired. Ray and Speck (1972a) found that *E. coli* gave more rapid maximum recovery from freeze injury in a complex nutrient medium such as trypticase soy broth supplemented with yeast extract than in the minimal glucose-salt medium. However, repair in inorganic phosphate buffer with or without $MgSO_4$ was also possible and occurred at pH values from 5 to 10 with an optimum between 8-9. The cells showed repair above 15° with maximum repair between 25 to 35° after a test period of 2 hours' incubation in the phosphate buffer. The injured cells were then found to be extremely sensitive to surface active agents common in selective media such as sodium desoxycholate and sodium lauryl sulfate, as well as to lysozyme (Ray and Speck, 1972b) and the repair process was demonstrated in the absence of DNA synthesis, protein synthesis, RNA synthesis and mucopeptide synthesis but found ATP synthesis to be essential. Similar finding was obtained by freezing *Salmonella anatum* (Ray *et al.*, 1972). Many of the

manifestations of injury resulting from heating and freezing, namely leakage of cellular material, increased sensitivity to selective media and recovery, are also exhibited following freeze-drying (Sinskey and Silverman, 1970; Ray *et al.*, 1971).

The most recent observations on injury are those of Jackson (1974). He found that storage of *Staphylococcus aureus* at 5° resulted in an increased sensitivity to a selective medium, mannitol-salt agar compared with a non-selective medium, trypticase soy agar. On transfer from 5 to 37° the cells rapidly recovered their ability to grow on mannitol-salt agar. Patterson (1974) confirmed these findings and also showed that storage of *E. coli* at 5° resulted in similar manifestations of injury.

The foregoing literature review was written with the intention of providing the reader with a general introduction to the temperature relationships of micro-organisms, together with a more detailed consideration of some of the more significant and interesting findings. The whole topic is too broad for exhaustive examination. However, it does illustrate quite clearly that biological systems exhibit many deviations from a linear response to temperature, and also that the activities of micro-organisms at temperatures other than the optimum are not necessarily either qualitatively or quantitatively related. In particular, there would appear to be a dearth of information on the effect of temperature between optimum and 0°. For food-borne pathogens and indicator organisms, this range is 37-0°. As large quantities of food are stored in this range, especially between 0-10°, it is surprising that this field has not received more attention. The present investigation was, therefore, undertaken in an attempt to provide some

data on the growth and viability of typical food-borne organisms in the range 0-37°.

MATERIALS AND METHODS

1. Temperature Gradient Studies

Test Organisms and Growth Media

Salmonella heidelberg, *Salmonella typhimurium* (B14512) and *Salmonella newport* were used in the study. The cultures of *S. heidelberg* and *S. newport* were obtained from the Provincial Laboratory of Public Health, Edmonton, Alberta, Canada. Stock cultures were maintained at 4° on tryptic soy agar and subcultured monthly.

Two media were used in the experiments: (1) a complex nutrient medium, tryptic soy broth (TSB) and (2) a minimal medium, glucose-salt broth (GSB) containing glucose 2.0 g; K_2HPO_4 7.0 g; KH_2PO_4 2.0 g; sodium citrate 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.1 g; $(NH_4)_2SO_4$ 1.0 g and distilled water to 1 liter. Glucose was sterilized by autoclaving separately from the salt solution and mixed after cooling.

The above media were adjusted to pH 5, 6 and 7 with pre-determined amounts of sterilized potassium hydroxide or sulphuric acid, or the pH was adjusted before autoclaving to give the final desired pH.

Preparation of Inoculum

The inoculum was prepared by subculturing the organism three times at the mid-logarithmic stage of growth at 37° (absorbance at 450 nm 0.35-0.4). The culture was then harvested by centrifugation at 5° (Sorvall RC2-B) and resuspended with the growth medium to absorbance 1.0. The growth medium of the inoculum was either TSB or GSB.

Measurement of Growth

0.1 ml of the prepared inoculum was placed into each of a series of optically matched tubes of 3/4 in. diameter containing 10 ml of either TSB or GSB (pH 5, 6 or 7). The tubes were then placed into the holes of a static temperature gradient incubator. The incubator consisted of an aluminum bar in which 6x16 holes were drilled at a 45-degree angle. A temperature gradient was created by heating the bar at one end and cooling at the other end with a compressor control circuit as designed by the Technical Service of the University of Alberta. The temperature exhibited by water in the various test tubes had been recorded and shown to remain constant within ±0.2° for at least 2 weeks. The temperatures to which the organisms were exposed were 0, 2, 4, 6, 9, 11, 14, 16, 19, 21, 23, 25, 28 and 30°. Samples were withdrawn for the measurement of absorbance and viable count. Absorbance of the culture was measured every few hours at 450 nm in a spectrophotometer (Bausch and Lomb Spectronic 20). Viable cell counts were determined at 0 time and every 5 days for 15 days by the pour-plate method using tryptic soy agar (TSA) as the plating medium. The plates were incubated for 24 hr at 37°. Dilution of samples was made in 0.1% peptone water at 0-4°. The pH of the culture was also measured with a Zeromatic II pH meter (Beckman Instruments Inc., Fullerton, California, U.S.A.) on the final day of incubation.

II. Effect of Low Temperature on *Salmonella heidelberg*

Test Organism and Growth Media

S. heidelberg was used throughout the study. Both tryptic soy broth and glucose-salt broth were used for the preparation of inoculum

and subsequent experimentation.

Plating media: (1) complex rich medium, tryptic soy agar + 0.5 % yeast extract (TSYA), (2) minimal medium, glucose-salt agar, (3) selective media: desoxycholate agar, MacConkey agar and brilliant green agar.

Glucose-salt agar was prepared by adding 1.5% of Bacto-agar to glucose-salt broth. All other media were supplied by Difco Laboratories Ltd., Detroit 1, Michigan, U.S.A. and all were sterilized as recommended by the manufacturer.

Preparation of Inoculum and Growth Condition

For the study of effect of suboptimal temperature on growth rate and ATP production, immediate temperature equilibration was desired. Also a cell concentration of absorbance 0.3 was required to facilitate the determination of ATP. Therefore, the inoculum was prepared by subculturing the organism three times at the mid-logarithmic stage of growth in glucose-salt broth at 37°, harvesting by centrifugation at 20° and resuspending with a small volume of growth medium. The inoculum was then put into 100-200 ml of medium in Erlenmeyer flasks pre-tempered to the desired temperature in a Metabolyte Refrigerated Water Bath Shaker (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey, U.S.A.) shaking at 175 rpm. The accuracy of the temperature control of this unit is ±0.2°. The determination of cell yield is described under the section, 'Yield Coefficient'.

For the study of cold injury at sub-minimal growth temperature, unless otherwise specified, inoculum was prepared by harvesting exponentially grown culture at 5° and resuspending in a small volume of growth medium. Incubation was carried out in the Metabolyte

Refrigerated Water Bath Shaker as described above or in a New Brunswick Psychrotherm incubator shaker (New Brunswick Scientific Co., New Jersey, U.S.A.) with a sensitivity of ±0.2°. When larger volumes of culture were required, such as for the determination of macromolecular composition of cells during storage, the inoculum was added to 1 litre of medium in a 2 l Erlenmeyer flask. The flask was fitted with a side arm and contained a Teflon coated stirring bar. Prior to inoculation, the flask of medium was placed in a 12"x12"x12" plexiglass tank fitted with a coolant circulating device that maintained the level of coolant in the plexi-glass tank above the level of medium in the flask. The coolant was stored in a separate water bath fitted with a heating and refrigeration system. The temperature of the coolant in the bath was controlled by a micro-set thermoregulator (Precision Scientific, Chicago, Illinois, U.S.A.) with a sensitivity of ±0.01°. Agitation of the medium in the flask was achieved by the use of a non-heating magnetic stirrer (Bellco Glass Inc., Vineland, New Jersey, U.S.A.). For the study of recovery of the cold-injured cells, unless otherwise specified, the cells were harvested by centrifugation at 2-5° and resuspended in cold recovery menstruum. Portions of the cell suspension were inoculated either into the recovery menstruum pretempered at the desired recovery temperature, or into cold recovery menstruum which was then incubated at the desired temperature.

Viable Count

Dilutions of samples were prepared by transferring 1 ml aliquots to screw capped test tubes containing 9 ml sterile 0.1% peptone water held at 0-4°. 0.1 ml of appropriate dilutions were then surface-plated in triplicate or duplicate on predried agar plates. The plates

were incubated at 37° for 24 hr and the colonies were enumerated. Further incubation did not increase the colony counts.

Plates of media were prepared by pouring approximately 12 ml sterile media into petri dishes in a Laminar flow hood (The Baker Co., Inc., Sanford, Maine, U.S.A.). The covers were left off the petri dishes for 20-30 min in the hood to ensure that the surface of the plates would be adequately dry before use. Poured petri dishes were inverted in stacks and stored in a cold room at 4° but were always used within 2 weeks of preparation.

Dry Weight

A 90 ml sample of the culture was harvested by centrifugation at 0° and resuspended in 6 ml 0.05 M phosphate buffer, pH 7.0. 5 ml of this suspension was transferred to a dried aluminium foil cup of known weight and the whole unit placed in a drying oven at 100° for 24 hr. The dried material was cooled to room temperature in a desiccator, weighed, and the dry wt/ml of the original culture established.

Yield Coefficient

Minimal medium minus sodium citrate containing 250 μg glucose/ml was placed in a 300 ml Erlenmeyer flask and inoculated with a washed suspension of cells from an exponentially growing culture (10% inoculum). The flask was incubated in a Metabolyte water-bath shaker at the desired temperature. The turbidity of the culture was followed until a constant reading was obtained. The increase in dry weight and residual glucose in the filtered medium were determined. The yield coefficient, K, was computed from the equation $K = (G-G_0)/C$, where G and G_0 are cell mass (μg dry weight/ml) at the end of the experiment

and at zero-time, respectively, and C is the glucose concentration in μg/ml at zero-time. Glucose was determined by the enzymatic Glucostat reagent (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.).

Determination of Nucleic Acids and Proteins

The preparation of bacterial samples for nucleic acid and protein analyses was carried out according to the procedure of Schmidt and Thanhausser (1945). 40 ml of culture was removed at the desired interval and centrifuged at 10,000 rpm for 10 min at 2°. The supernatant was discarded and the pellet was washed twice in 0.1 M K_2HPO_4/KH_2PO_4 buffer of pH 7 (0-4°) and finally resuspended in 30 ml of the phosphate buffer. The protein and nucleic acid content was then determined as follows:

Protein

The protein content of cells was determined colorimetrically after extraction with alkali according to the method of Lowry *et al.* (1951). 5 ml of the buffered suspension was centrifuged for 8 min at 10,000 rpm in a 12 ml conical centrifuge tube. The cells were resuspended in 1.5 ml of 1N NaOH and the tube capped with a marble and placed in a water bath at 37° for 2 hr. The tube of digest was either sealed with a paraffin film for storage in the refrigerator (4°) until assay within 2 weeks or estimated immediately. The actual estimation involved 0.5-1.0 ml of the alkaline digest + 5 ml of reagent D (prepared by mixing 2% Na_2CO_3, 1% $CuSO_4 \cdot 5H_2O$ and 2% sodium tartrate in the ratio of 100:1:1) which, after 10 min at room temperature, was supplemented with 0.5 ml of Folin-Ciocalteau reagent (diluted 1:1 with water). The tubes were left at room temperature for a further 30 min when the absorbance at 660 nm was measured. A standard curve was

prepared using bovine serum albumin (Calbiochem).

Nucleic acids

The remaining 25 ml portion of the buffered suspension was centrifuged and washed once with 10 ml 0.2 N $HClO_4$ at 0-4°. After centrifugation the cells were resuspended in 5 ml 0.3 N NaOH and incubated in capped tubes in a water bath at 37° for 70 min with mixing once or twice during incubation. After cooling to 0°, to facilitate maximum precipitation on addition of acid, 3 ml of cold 1.2 N $HClO_4$ was added, and the preparation allowed to stand in ice for a further 20 min. The samples were centrifuged and the resulting supernatant transferred to a test tube for RNA estimation. The precipitate was washed with 10 ml cold 0.2 N $HClO_4$, centrifuged and the supernatant added to the RNA sample.

The resulting precipitate was dissolved in 3 ml 0.5 N $HClO_4$ at 80° for 20 min. This preparation was then centrifuged, and 1 ml aliquots of the supernatant used for the DNA estimation, by a slight modification of the Burton (1956) procedure.

The RNA content was determined by the orcinol method (Schneider, 1957) using the 1-2 ml aliquots from above +2 ml 0.2 N $HClO_4$ and adding to this 4 ml $FeCl_3$/HCl reagent (0.1% $FeCl_3 \cdot 6H_2O$ in conc. HCl). This was mixed and 0.4 ml orcinol reagent added (10% orcinol in 95% ethanol). The mixture was heated in capped test tubes for 30 min in a boiling water bath, and the intensity of the green color read at 660 nm in a DB-G spectrophotometer. A standard curve was prepared using D-ribose (Calbiochem).

The 1 ml aliquots for the DNA determination were mixed with 2 ml diphenylamine reagent, (1 g diphenylamine in 100 ml of glacial acetic

acid plus 2.75 ml H_2SO_4 supplemented with 0.5 ml of aqueous acetaldehyde solution, 16 mg/ml, immediately before use). The tubes, again capped with marbles, were kept at 30° for 16 hr. after which the absorbance was measured at 600 nm (Burton, 1956).

ATP Determination

Samples were prepared by pipeting 7 ml cell culture in glucose-salt medium into 0.4 ml 70% $HClO_4$. After mixing and holding at room temperature for 20 min, the samples were cooled in an ice-bath for 15 min. After centrifugation the supernatant was neutralized by titration with a solution 0.5 M with respect to triethanolamine and 6N with respect to K_2CO_3 using a combination pH electrode (Fisher Scientific Co., Ltd., Pittsburgh, PA., U.S.A.). The neutralizing solutions were added slowly and with constant mixing in order to avoid localized regions of alkaline pH. The pH of the neutralized sample was between 6.5 and 7.0. Following a 15 min storage period in ice, the samples were centrifuged to remove potassium perchlorate. The clear supernatant was stored in an ice-bath until used for the assay of ATP using the firefly luciferin-luciferase system essentially as described by Cole *et al.* (1967). Worthington freeze-dried firefly extract, which contained 20 mM magnesium sulfate and 50 mM potassium arsenate buffer when reconstituted, was suspended in water at a concentration of 10 mg/ml. The suspension was allowed to stand for 10 hr at 0°; it was then centrifuged and the supernatant solution was used for the assay. The following aliquots were added to an acid-cleaned glass scintillation vial: 2 ml of a buffer solution containing 0.1 M $MgSO_4$ and 0.025 M glycylglycine (pH 7.5), and 10-100 μliters of the ATP sample; 50 μliters of firefly extract. Within 7 sec of adding and

mixing the firefly enzyme, the vial was introduced into the counting chamber of a Unilux II Nuclear-Chicago liquid scintillation counter and the light flashes were counted for three successive periods of 12 sec. The first count of 12 sec was usually high and was discarded; the second and third counts were close and were averaged. The scintillation counter channel was set as for tritium counting (maximal amplifier gain in the attenuation, upper gate 9.9v, lower gate 0.5 v), and the photomultiplier coincidence circuit was switched out. A standard curve was prepared using ATP (sodium salt, 98% pure, Sigma). Figure 1.

Fluorimetry

Altered permeability of cold-injured cells was demonstrated by the ability of 8-anilino-1-naphthalene sulphonic acid (ANS) to penetrate the cells. Aqueous solutions of ANS do not fluoresce when excited by ultraviolet light; in the presence of protein the dye combines with negatively-charged groups and the conjugate fluoresces strongly when excited by light of wavelength 346 millimicrons (Weber and Laurence, 1954). When washed cells were suspended in dilute solutions of this dye no fluorescence was observed, indicating there were no groups on the surface of the cell with which the dye could combine. Treatment with a membrane-binding antibiotic, however, resulted in increasing fluorescence, presumably the alteration in cellular permeability allowing the dye to penetrate the cell and combine with cell protein (Newton, 1954). ANS has also been shown to form a fluorescent complex with *Aerobacter aerogenes* when the osmotic barrier is destroyed (Matthews and Sistrom, 1960; Strange and Postgate, 1964; Allwood and Russell, 1968).

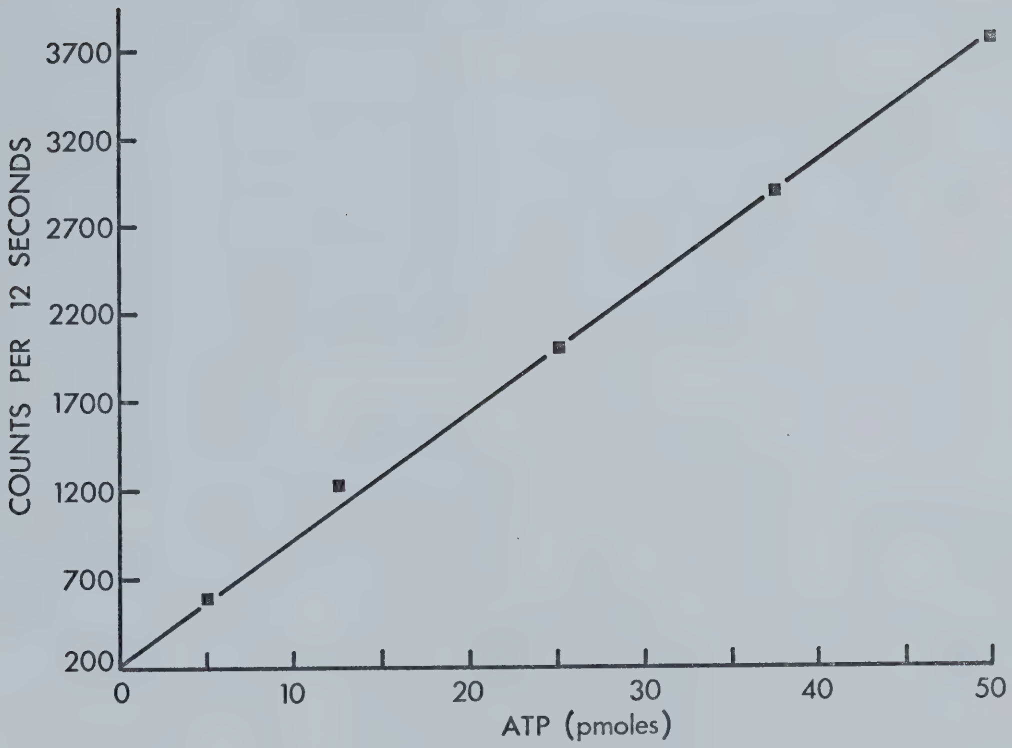

FIG. 1. Standard curve for the estimation of ATP.

Intensities of fluorescence were measured in a Turner III Fluorometer (G.K. Turner and Assoc., Palo Alto, California, U.S.A.), fitted with a 405 filter giving an exciting wavelength of approximately 405 millimicrons, and a No. 2 filter giving a recording wavelength greater than 480 millimicrons.

Ten ml of culture (10^8 cells/ml) was harvested and 4 ml of ANS (Eastman Kodak Co., Rochester, New York, U.S.A.) aqueous solution with a concentration of 5 mM was added with mixing. The suspension was allowed to stand at room temperature for 15 min before measurement of the fluorescence.

Determination of Injury and Recovery

The various states of the bacterial cells after cold storage were defined according to Iandolo and Ordal (1966): (i) the number of injured cells was determined by the difference in viable counts on a complex medium and the count on a selective medium; (ii) uninjured cells were those which grew on the selective medium after cold storage; (iii) the number of dead cells was the difference between the viable count on the complex medium before and after cold storage. Recovered cells were those injured cells which regained their ability to grow on the selective medium.

Effects of Inhibitors on Viability and Recovery

Inhibitors of DNA, RNA, protein, cell wall and ATP synthesis were incorporated into the storage and recovery menstrua so as to elucidate what biosynthetic process or processes were involved in the recovery of the ability of injured cells to grow on selective medium. The following antibiotics and metabolic inhibitors were used: chloramphenicol

100 μg/ml, hydroxyurea 500 μg/ml, 2,4-dinitrophenol 80 μg/ml, oligomycin 10 μg/ml, gramicidin 10 μg/ml, Penicillin G 100 μg/ml, D-cycloserine 50 μg/ml (Sigma Chemical Co., St. Louis, Missouri, U.S.A.); fluorouracil 100 μg/ml, rifamycin 10 μg/ml, rifampin 10 μg/ml, nalidixic acid 10 μg/ml (Calbiochem, San Diego, California, U.S.A.); sodium cyanide 100 μg/ml and sodium azide 500 μg/ml (Fisher Scientific Co., Fairlawn, New Jersey, U.S.A.).

RESULTS

I. Effect of Suboptimal Temperatures on the Growth and Viability of *Salmonella*.

Although *Salmonella* spp. normally have an optimum growth temperature of 37°, they can survive and even increase in number at much lower temperatures (Matches and Liston, 1968). However, the amount of information in this area is very limited. The purpose of this study was initially to determine the pattern of growth and viability of *Salmonella* incubated at sub-optimal growth temperatures. By using the temperature gradient incubator, small temperature gradients and long incubation times were made possible. *S. heidelberg, S. typhimurium* and *S. newport* were used in the study as they are among the strains most frequently isolated from human sources (Morbidity and Mortality Reports, 1965).

A. Growth and Viability in Tryptic Soy Broth (Temperature Gradient Incubator)

The effect of temperature and pH on the growth of *S. heidelberg* in tryptic soy broth at pH 5, 6 and 7 was followed by measuring absorbance at 450 nm. The results of this preliminary study are shown in Figs. 2, 3 and 4. At any particular pH level it can be seen that the higher the incubation temperature, the faster was the rate of growth and in most cases, the higher was the population density. The most noticeable difference between pH level is seen in the maximum population density. At pH 7 the absorbance always exceeded 1.0 when growth occurred, but at pH 6 the maximum absorbance was 1.0 or less, and at pH 5 the maximum

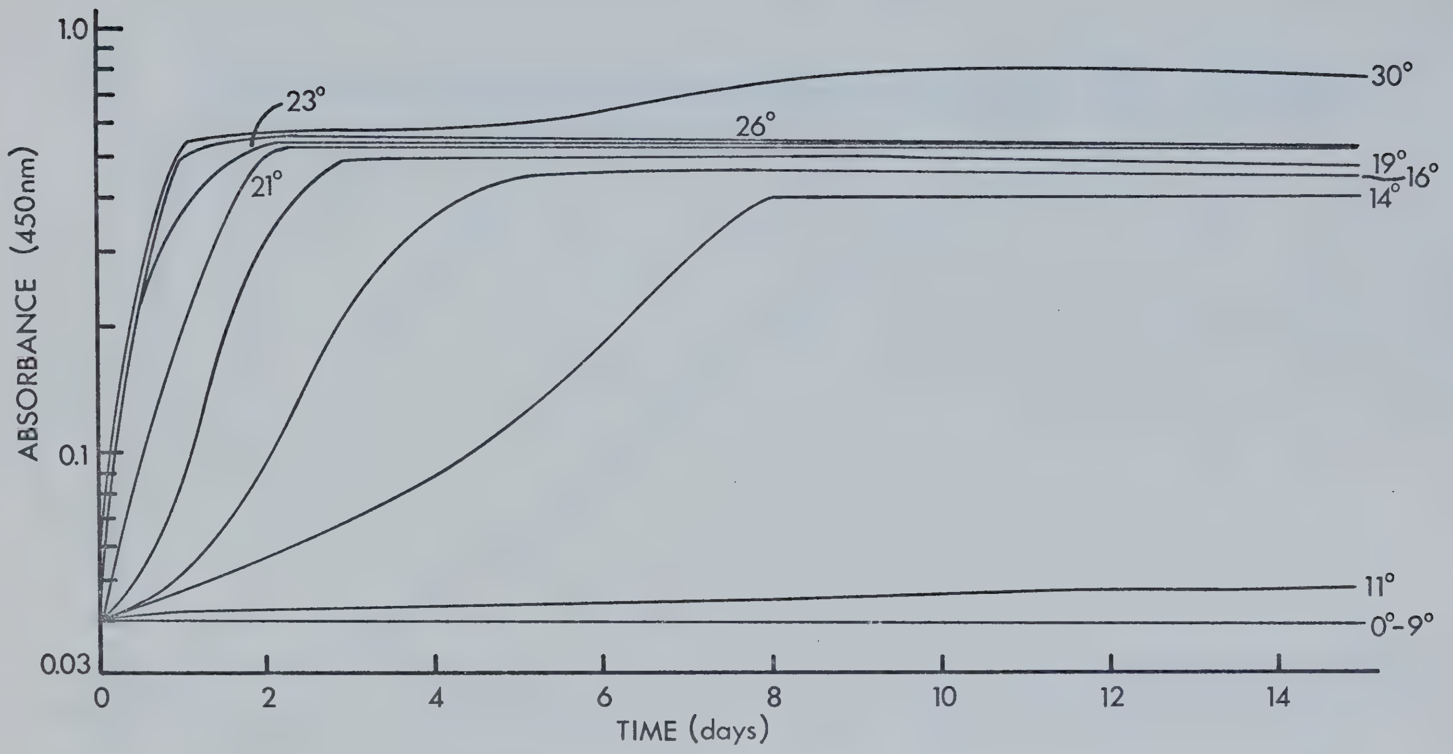

FIG. 2. Effect of temperature on the growth of *Salmonella heidelberg* in tryptic soy broth at pH 5.

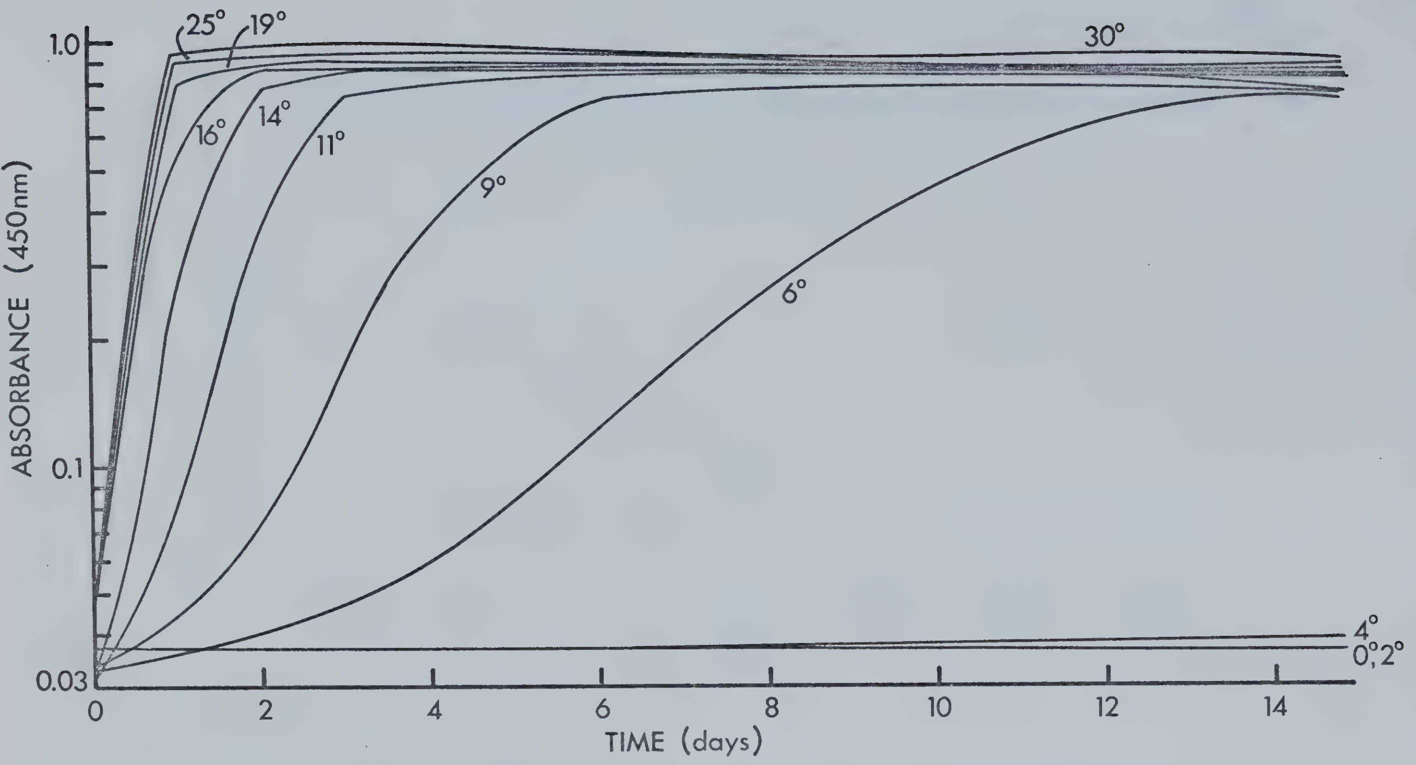

FIG. 3. Effect of temperature on the growth of *Salmonella heidelberg* in tryptic soy broth at pH 6.

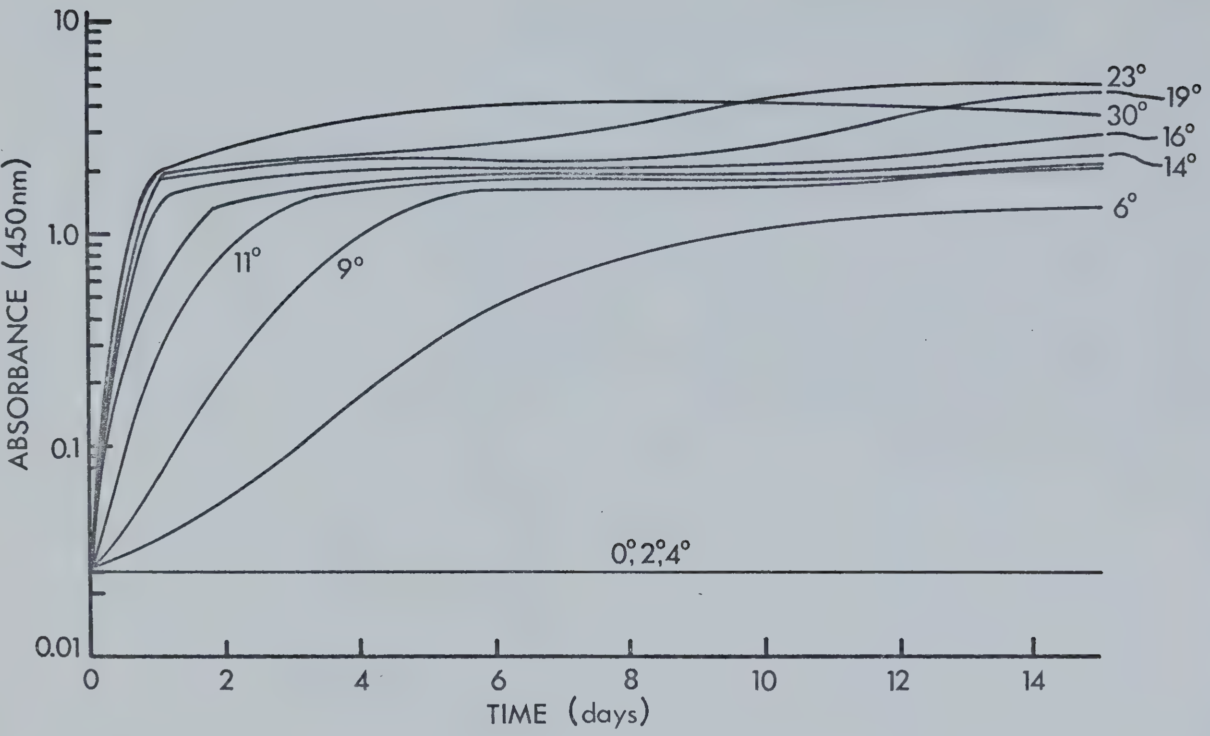

FIG. 4. Effect of temperature on the growth of *Salmonella heidelberg* in tryptic soy broth at pH 7.

absorbance did not exceed 0.8 units.

The minimum temperature at which growth could be initiated was 6° at pH 6 and 7 and 14° at pH 5.

The results on the viability of *S. heidelberg* grown at pH 5, 6 and 7 are shown in Figs. 5, 6 and 7. In accordance with the results on absorbance, the highest number of viable cells was found at pH 7. The growth rate increased with increasing temperature at pH 5, 6 and 7. A most interesting observation is the loss of viability at pH 5 and pH 7. In both cases the loss of viability was most rapid at the higher incubation temperatures. This is most apparent in Fig. 7 where it can be seen that the loss of viability is more rapid with increasing incubation temperature. In contrast the viability of cultures grown at pH 6 was maintained at almost the maximum level until the end of the experiment, even at the higher incubation temperature.

The final pH of the broth cultures was determined to see if different incubation temperatures had an effect on the gross metabolism of the cultures. The results are presented in Table 1. In most cases where growth occurred at pH 7 the final pH of the culture was more alkaline than the initial pH. In contrast the final pH of cultures grown at pH 5 and 6 were more acidic than the initial pH.

The behavior of *S. typhimurium* and *S. newport* exposed to the same experimental conditions were very similar to those of *S. heidelberg* (Figs. 8-19 and Tables 2 and 3).

B. Growth and Viability in Glucose-Salt Broth (Temperature Gradient Incubator)

This experiment was undertaken to compare the growth response in a simple medium, glucose-salt broth (GSB) with growth in the complex

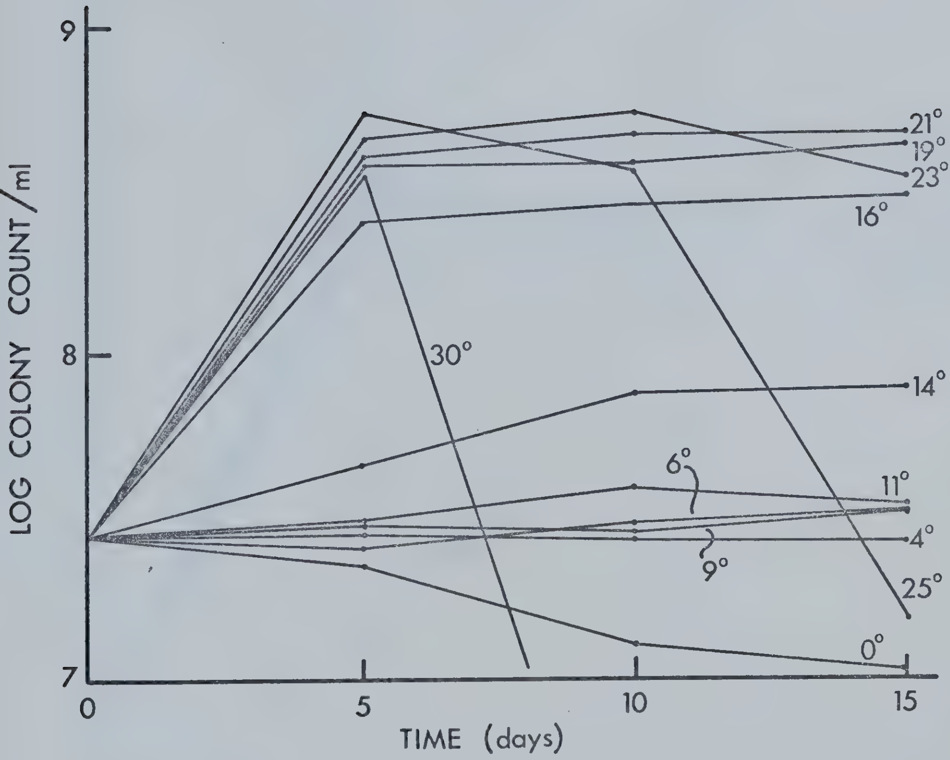

FIG. 5. Effect of temperature on the growth and viability of *Salmonella heidelberg* in tryptic soy broth at pH 5.

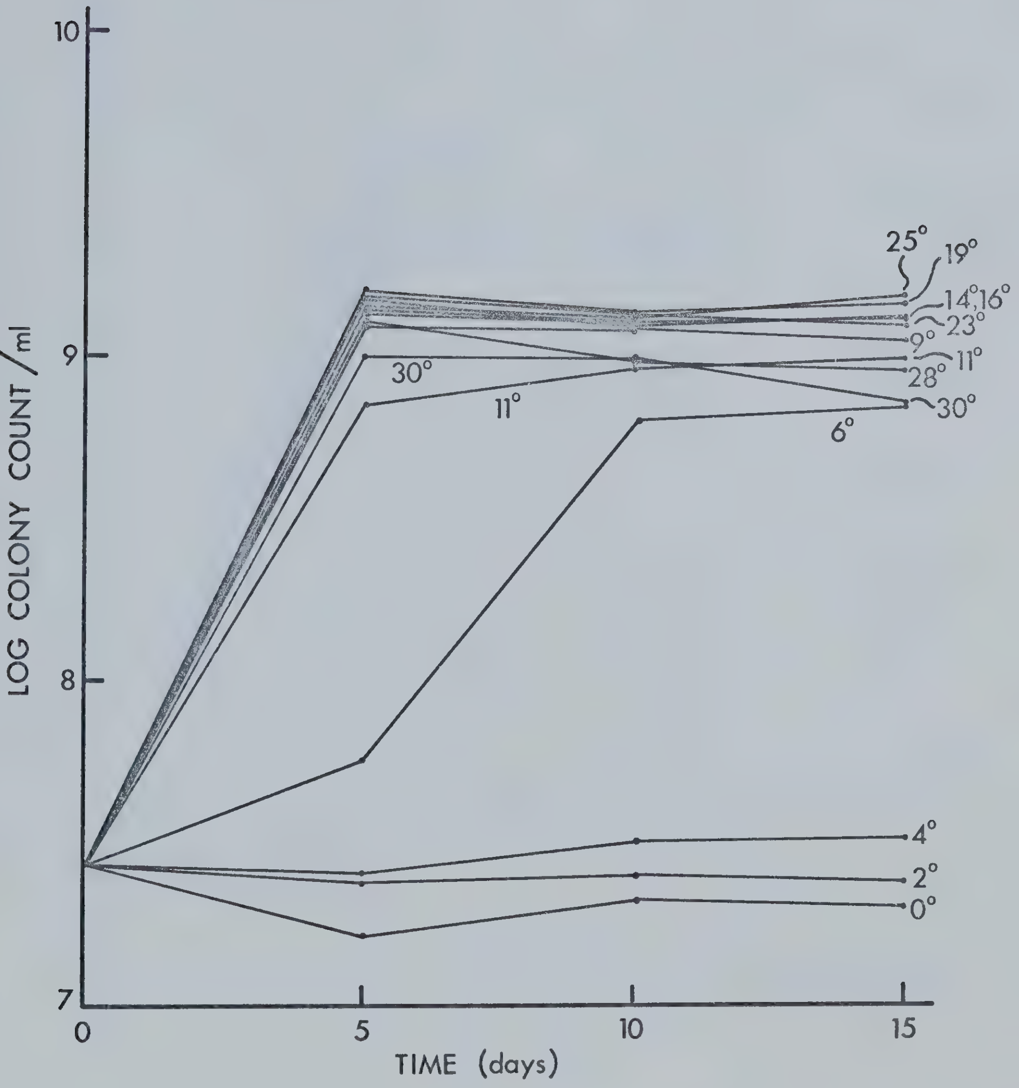

FIG. 6. Effect of temperature on the growth and viability of *Salmonella heidelberg* in tryptic soy broth at pH 6

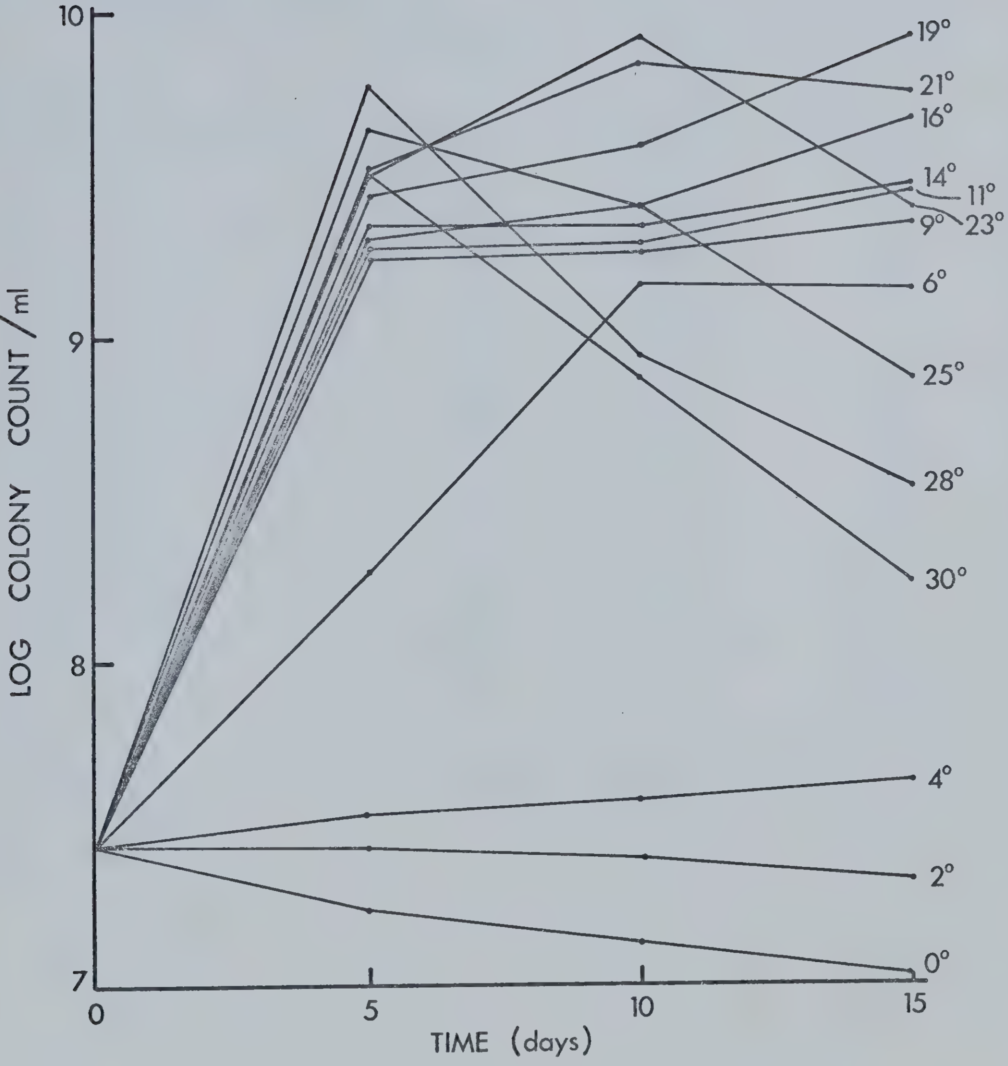

FIG. 7. Effect of temperature on the growth and viability of *Salmonella heidelberg* in tryptic soy broth at pH 7.

TABLE 1. Final pH of cultures of *Salmonella heidelberg* after growth at different temperatures in tryptic soy broth, pH 5, 6 and 7.

	Final pH after growth at an initial pH of		
Temperature	5	6	7
0	5.0	6.0	7.0
2	5.0	6.0	7.0
4	5.0	6.0	7.0
6	5.0	5.5	6.0
9	5.0	4.9	6.3
11	5.0	5.0	6.7
14	4.9	5.1	7.0
16	4.7	5.2	7.5
19	4.7	5.2	8.2
21	4.6	5.3	8.1
23	4.6	5.3	8.4
25	4.6	5.3	8.6
28	*n.d.*	5.3	8.7
30	4.5	5.3	8.8

n.d. - not done.

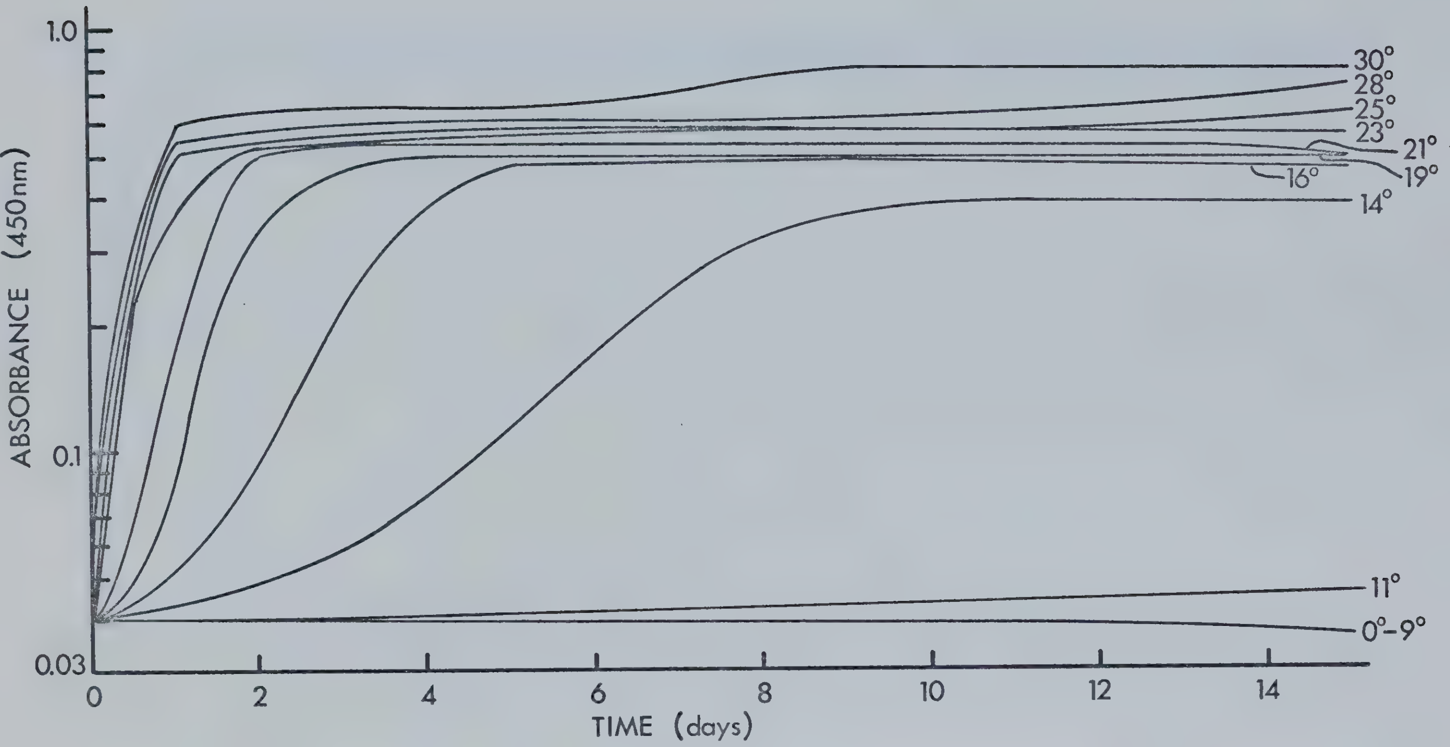

FIG. 8. Effect of temperature on the growth of *Salmonella typhimurium* in tryptic soy broth at pH 5.

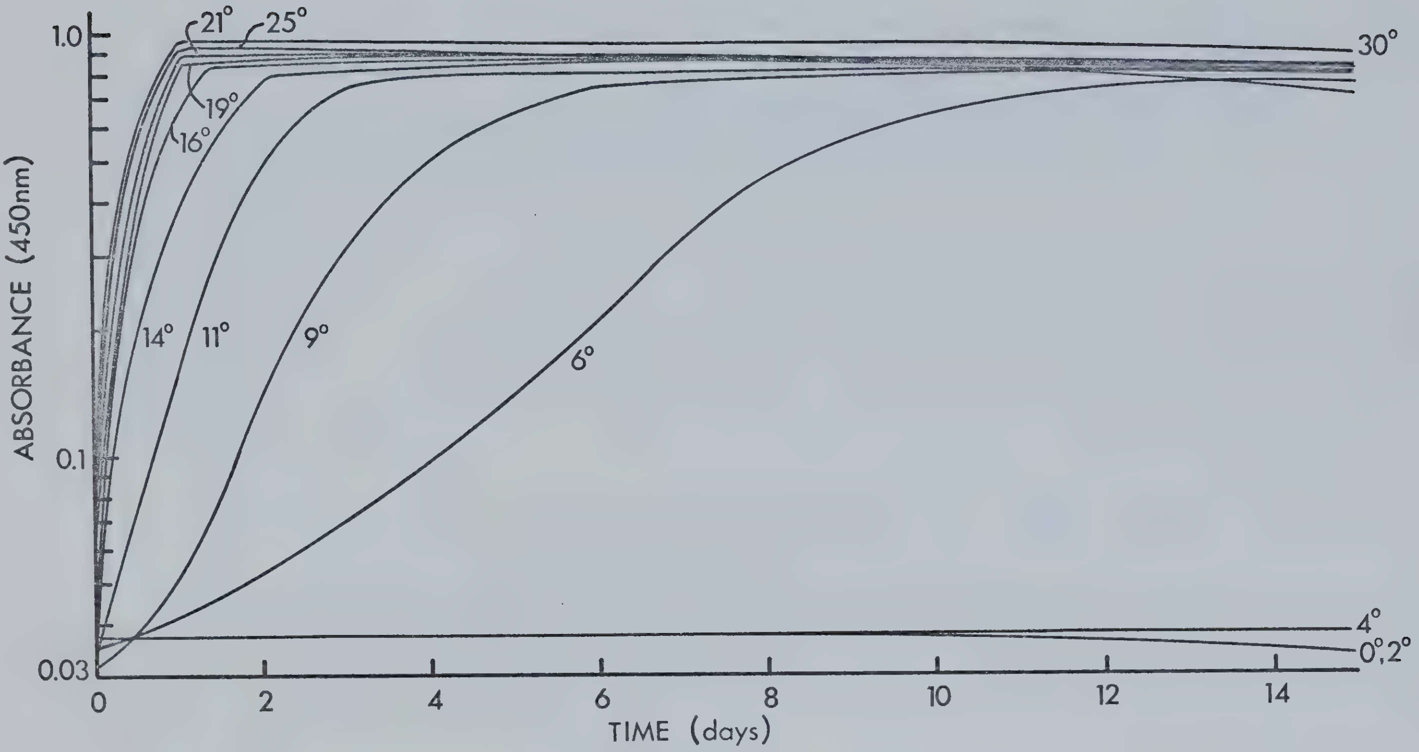

FIG. 9. Effect of temperature on the growth of *Salmonella typhimurium* in tryptic soy broth at pH 6.

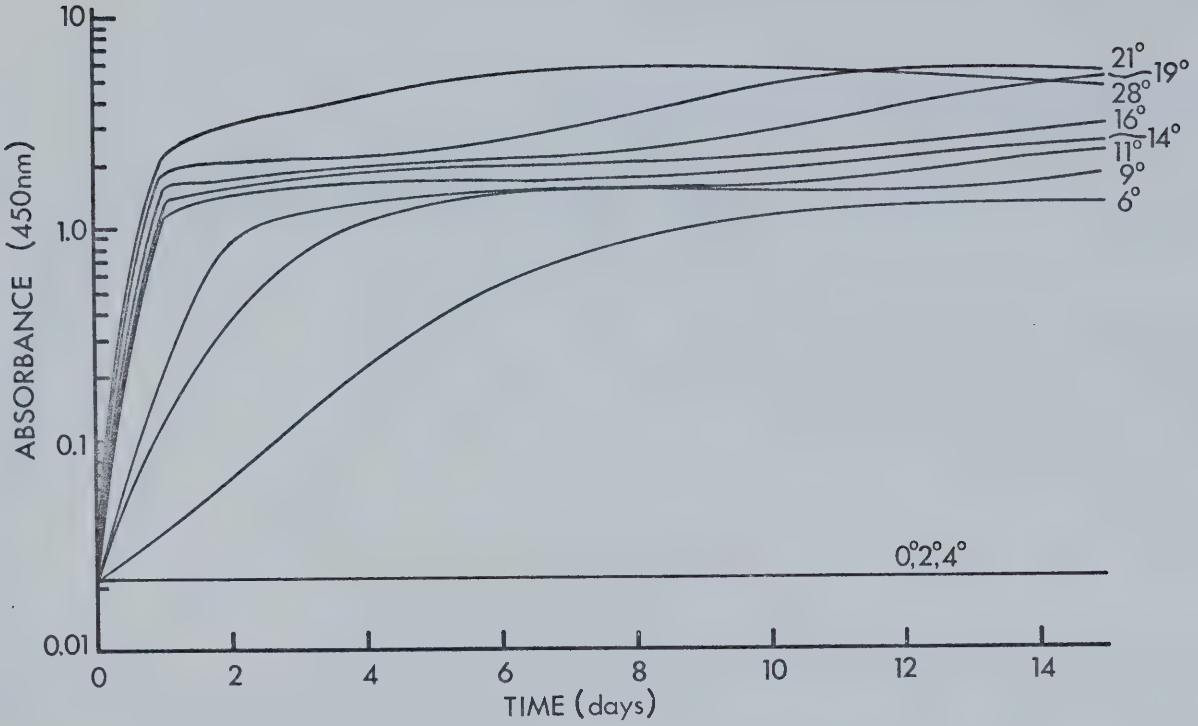

FIG. 10. Effect of temperature on the growth of *Salmonella typhimurium* in tryptic soy broth at pH 7.

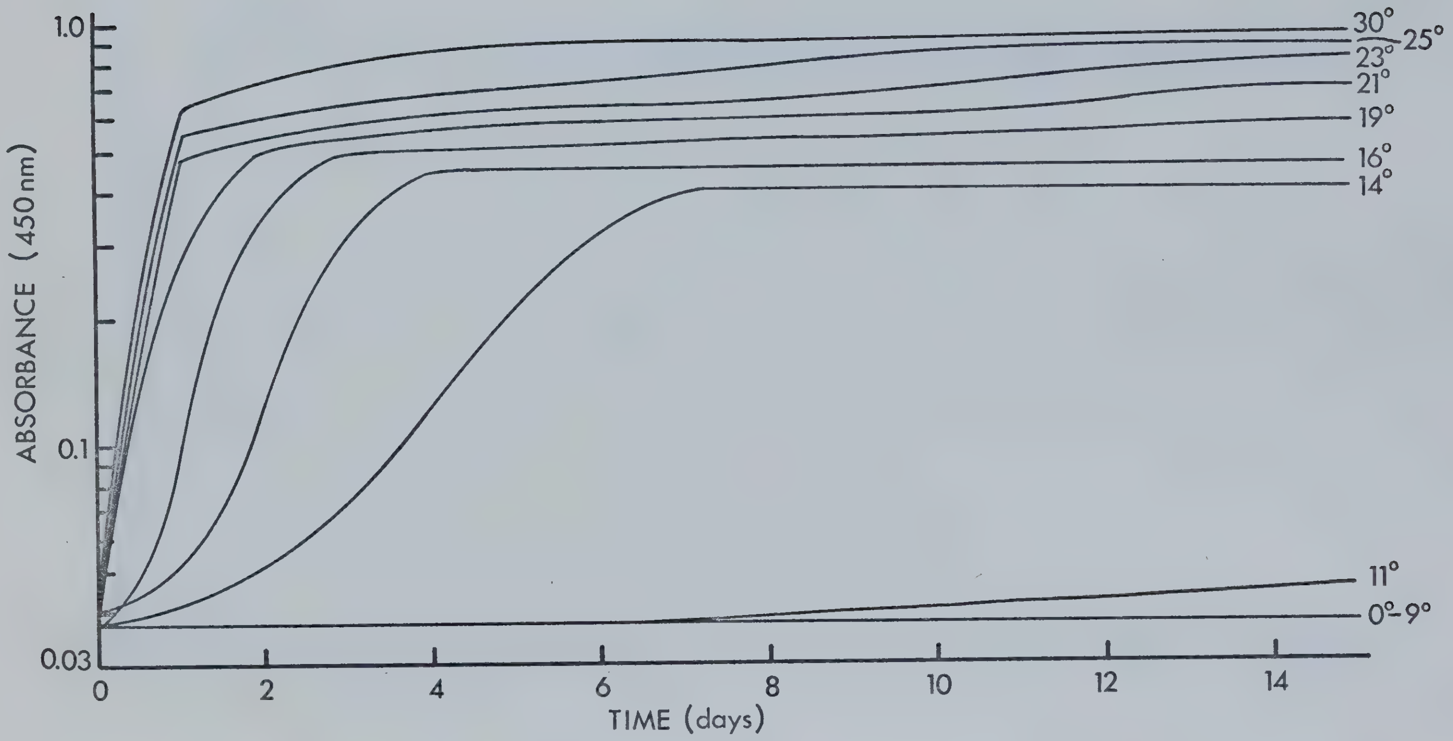

FIG. 11. Effect of temperature on the growth of *Salmonella newport* in tryptic soy broth at pH 5.

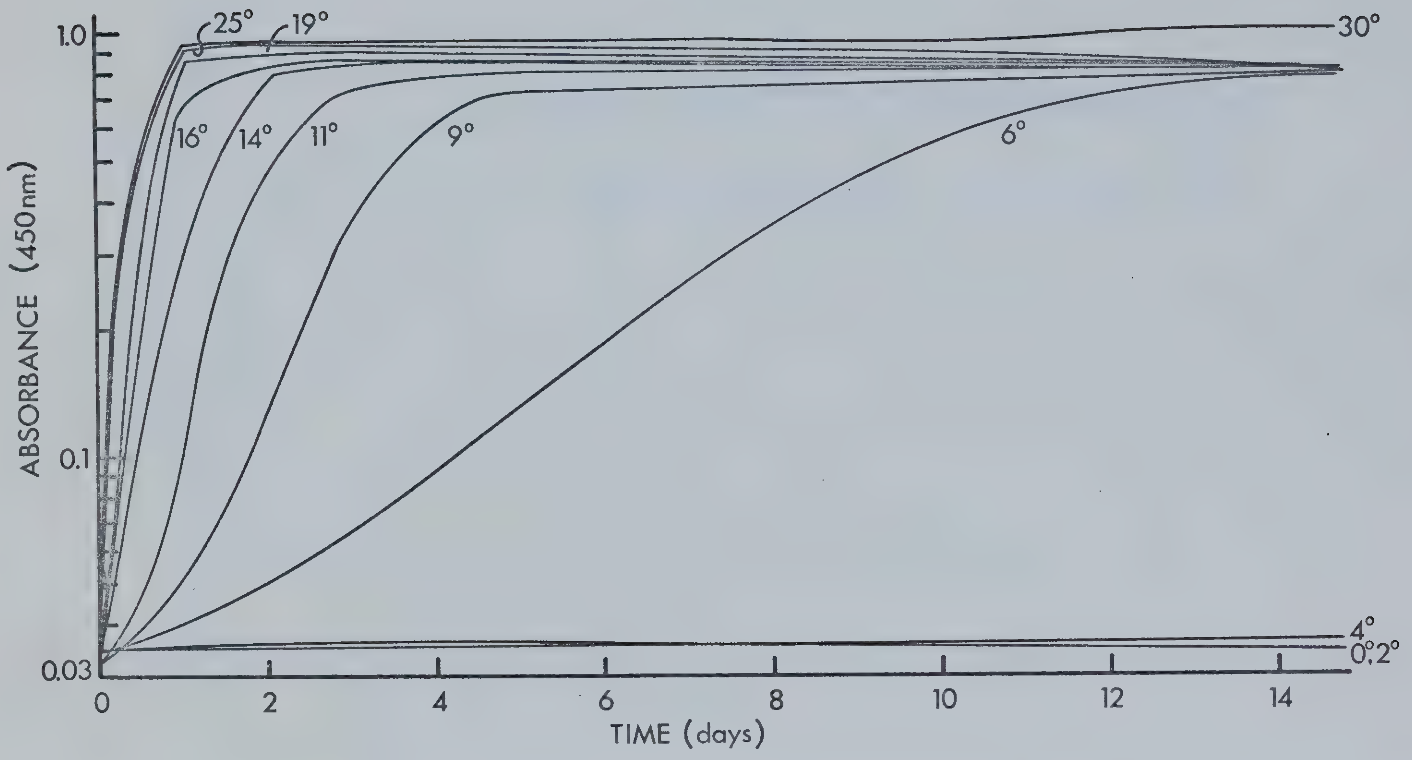

FIG. 12. Effect of temperature on the growth of *Salmonella newport* in tryptic soy broth at pH 6.

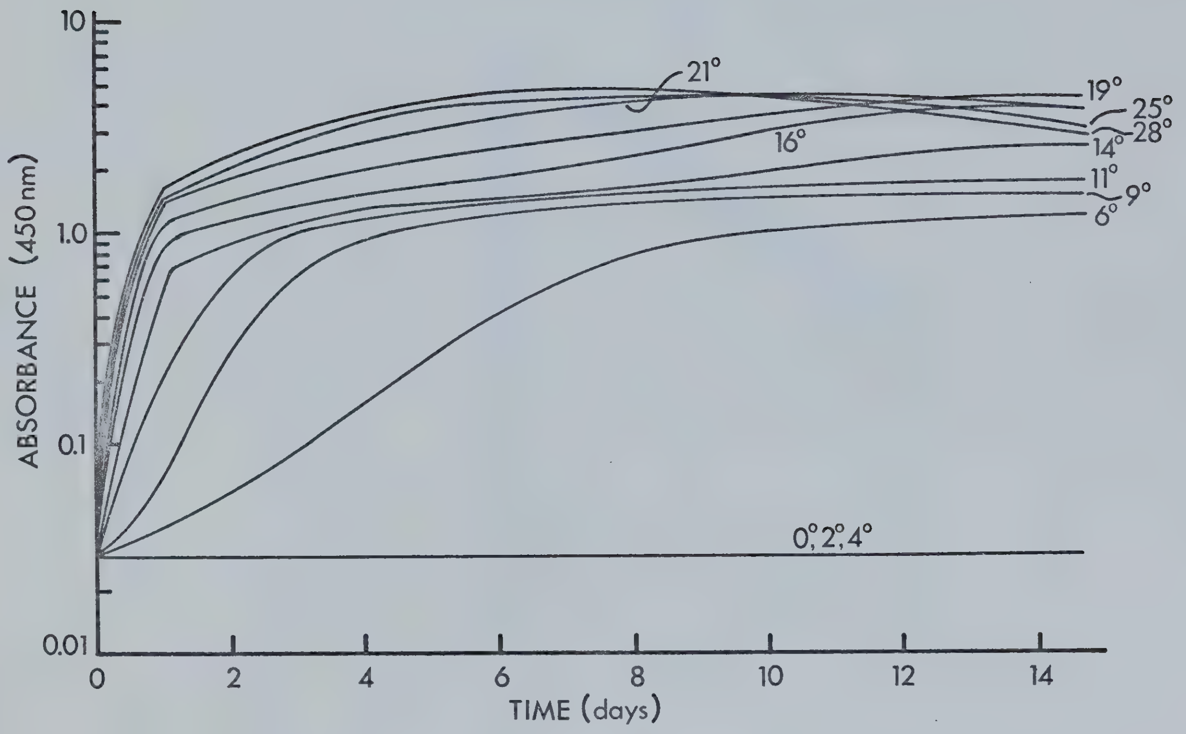

FIG. 13. Effect of temperature on the growth of *Salmonella newport* in tryptic soy broth at pH 7.

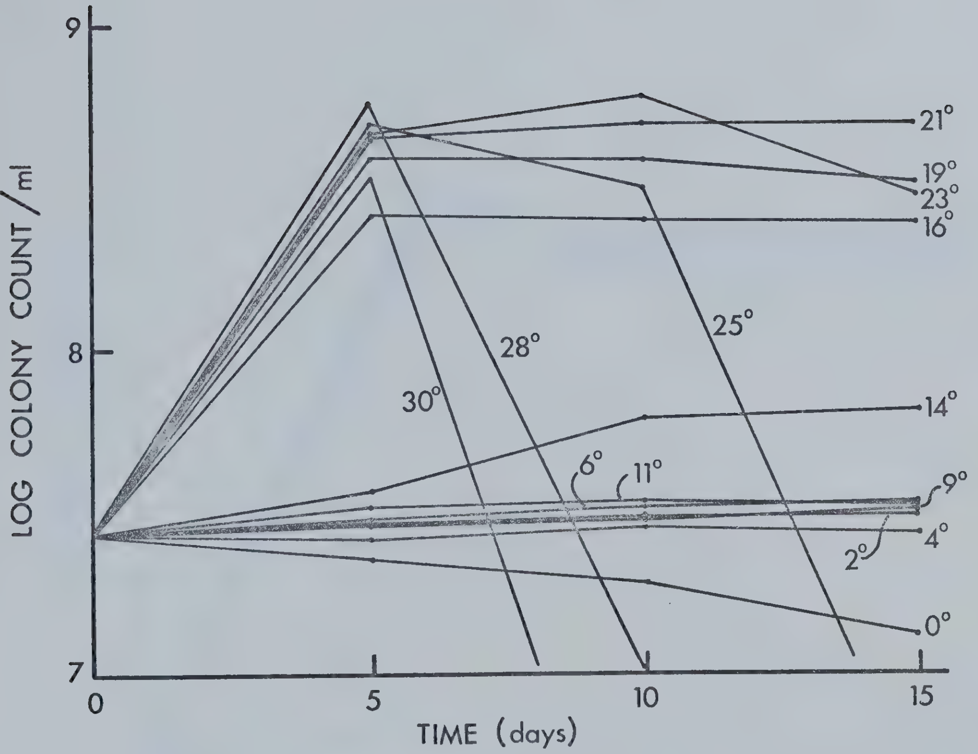

FIG. 14. Effect of temperature on the growth and viability of *Salmonella typhimurium* in tryptic soy broth at pH 5.

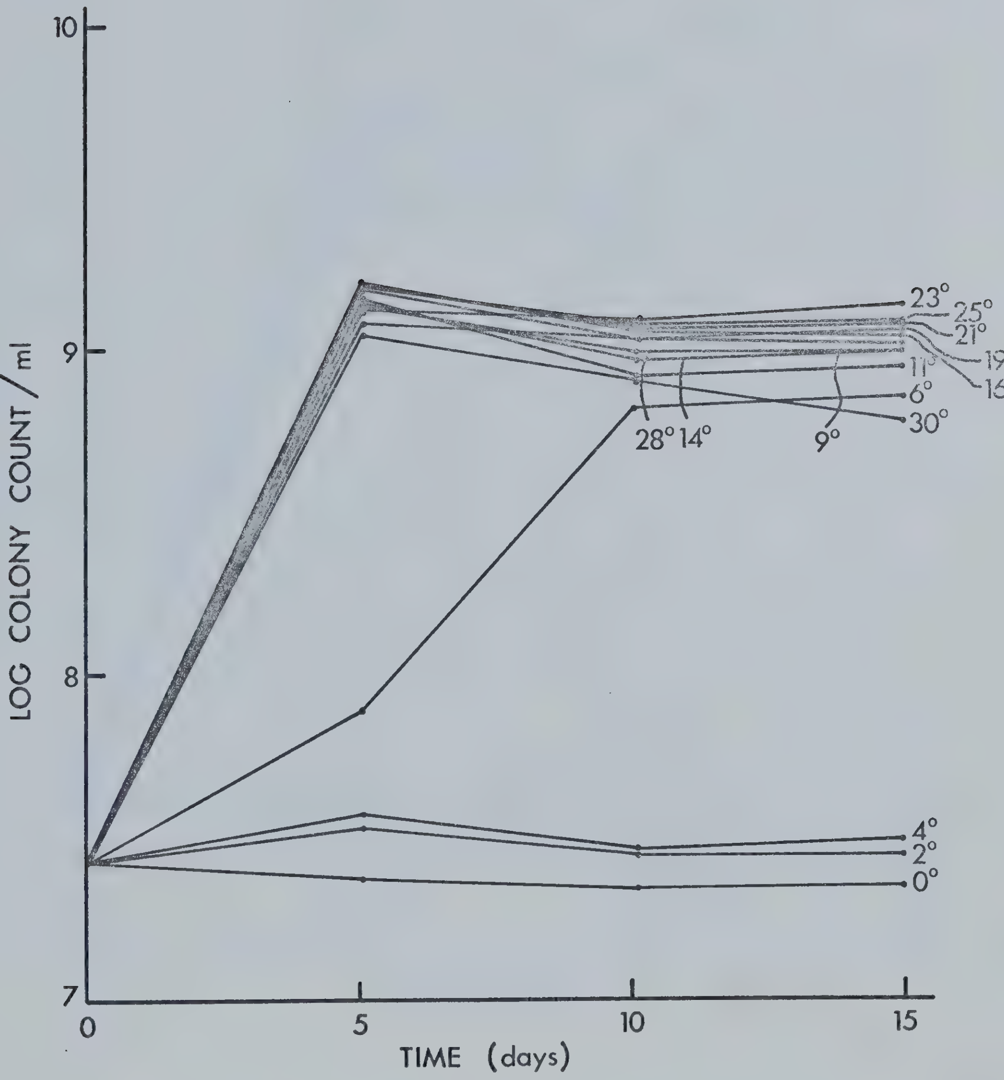

FIG. 15. Effect of temperature on the growth and viability of *Salmonella typhimurium* in tryptic soy broth at pH 6.

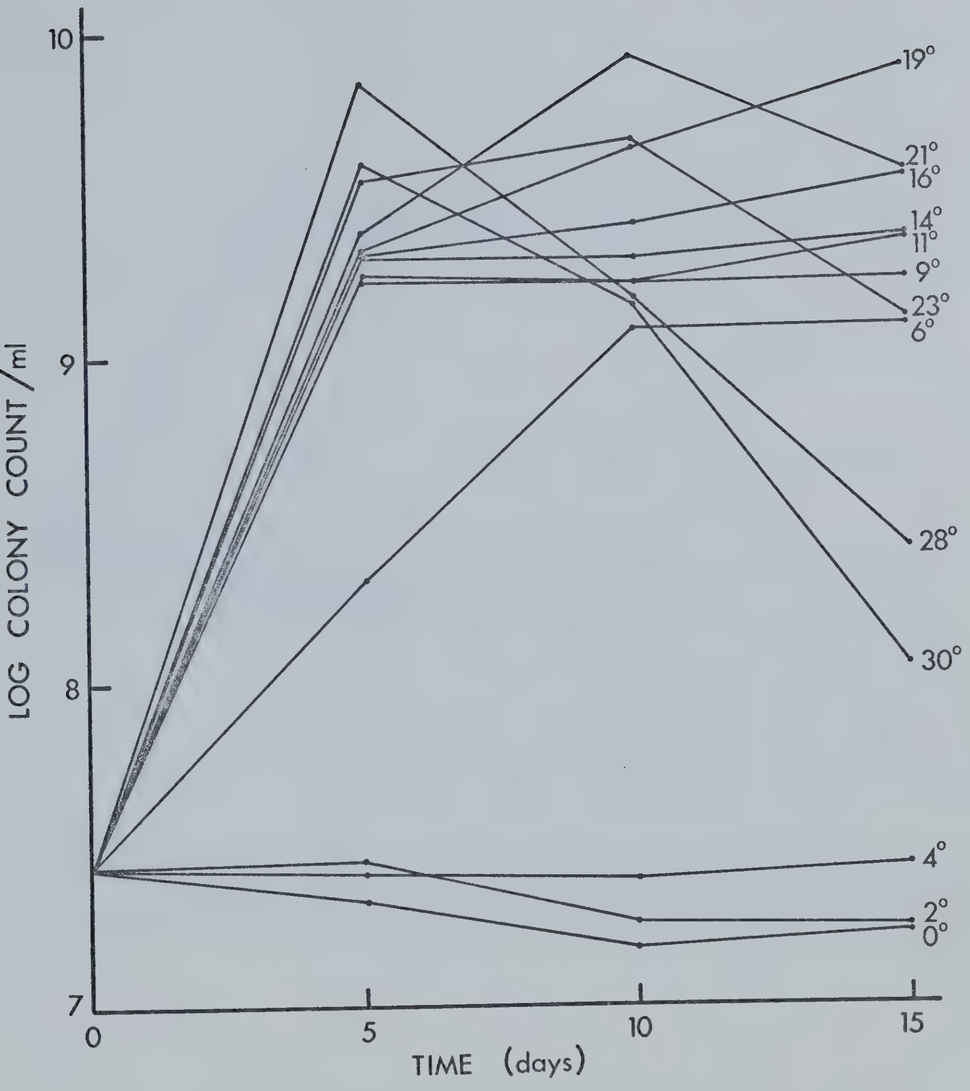

FIG. 16. Effect of temperature on the growth and viability of *Salmonella typhimurium* in tryptic soy broth at pH 7.

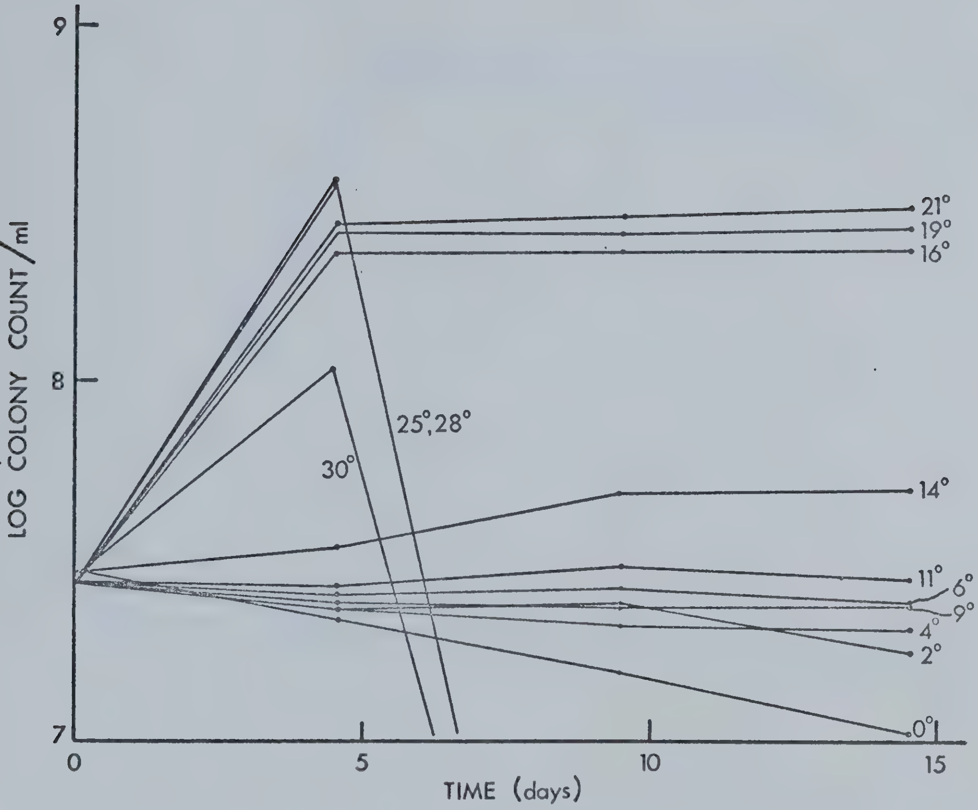

FIG. 17. Effect of temperature on the growth and viability of *Salmonella newport* in tryptic soy broth at pH 5.

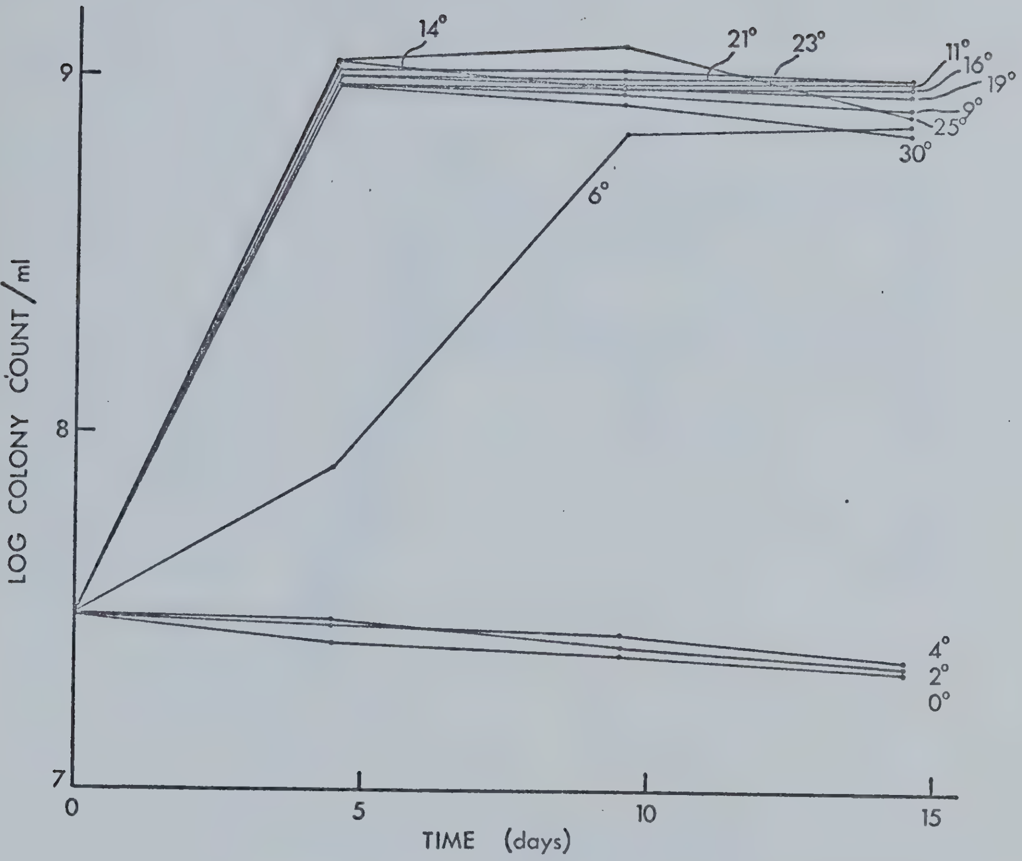

FIG. 18. Effect of temperature on the growth and viability of *Salmonella newport* in tryptic soy broth at pH 6.

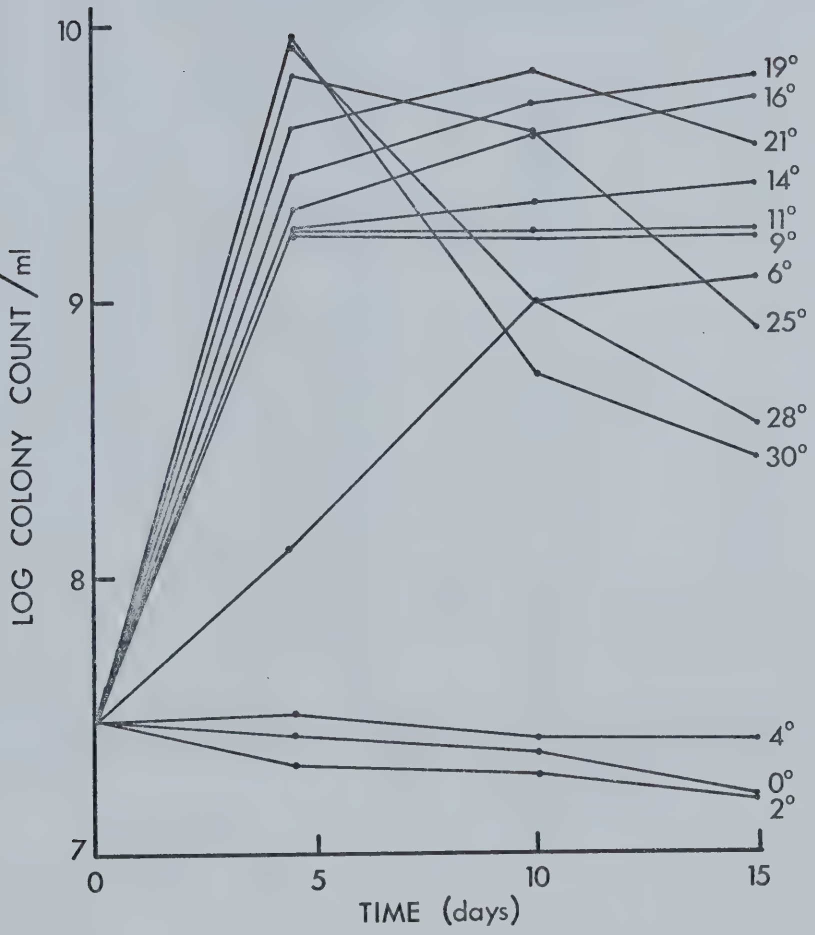

FIG. 19. Effect of temperature on the growth and viability of *Salmonella newport* in tryptic soy broth at pH 7.

TABLE 2. Final pH of cultures of *Salmonella typhimurium* after growth at different temperatures in tryptic soy broth, pH 5, 6 and 7.

Temperature	Final pH after growth at an initial pH of		
	5	6	7
0	5.0	6.0	7.0
2	5.0	6.0	7.0
4	5.0	6.0	7.0
6	5.0	5.5	6.1
9	5.0	5.2	6.3
11	5.0	5.1	6.7
14	4.9	5.3	6.9
16	4.8	5.4	7.6
19	4.7	5.5	8.1
21	4.6	5.4	8.3
23	4.6	5.4	8.4
25	4.6	5.5	*n.d.*
28	4.6	5.4	8.7
30	4.5	5.4	8.7

n.d. - not done.

TABLE 3. Final pH of cultures of *Salmonella newport* after growth at different temperatures in tryptic soy broth, pH 5, 6 and 7.

Temperature	Final pH after growth at an initial pH of		
	5	6	7
0	5.0	6.0	7.0
2	5.0	6.0	7.0
4	*n.d.*	6.0	7.0
6	5.0	5.3	6.0
9	5.0	4.7	6.7
11	5.0	4.9	7.0
14	4.8	4.9	7.5
16	4.6	5.0	8.0
19	4.5	5.1	8.2
21	4.5	5.2	8.3
23	4.4	5.2	8.4
25	4.3	5.3	8.6
28	4.4	5.3	8.7
30	4.4	5.3	8.8

n.d. - not done.

medium, tryptic soy broth (TSB). In view of the similar results for all three cultures in the previous experiment only *S. heidelberg* was used.

Glucose-salt broth gave much slower and less total growth than tryptic soy broth at all temperatures and all levels of pH. The results for absorbance (Figs. 20, 21 and 22), viable counts (Figs. 23, 24 and 25) and final pH (Table 4) all indicated that the minimal temperatures at which growth occurred in GSB at pH 5, 6 and 7 were 11°, 9° and 9° respectively. The cultures grown at pH 5 and 6 died off rapidly in the stationary phase at temperatures above 21° (Figs. 23 and 24) and the final pH of the cultures were around 4.2 and 4.6 respectively (Table 4). On the other hand, cultures grown at pH 7 survived equally well both at high and low temperatures of incubation (Fig. 25) resulting in a final pH around neutrality (Table 4).

II. Effect of Suboptimal Temperatures on Growth Rate, ATP Synthesis and Cell Yield of *Salmonella heidelberg*.

Frank *et al.* (1972) studied several properties of psychrophilic pseudomonads grown in batch culture in nutrient broth at 2 and 30°. No differences was observed in the size, catalase activity, deoxyribonucleic acids, ribonucleic acid or protein content of cells grown at either temperature. Investigation in this Department also found that there was no significant difference in the macromolecular composition of *S. typhimurium* grown at 10 and 37° (Hsu, 1972). On the other hand, there is a dearth of information on the effect of temperature on ATP synthesis and cell yield.

S. heidelberg was used in the study and the culture medium was glucose-salt broth (GSB). An exponentially growing culture at 37° was

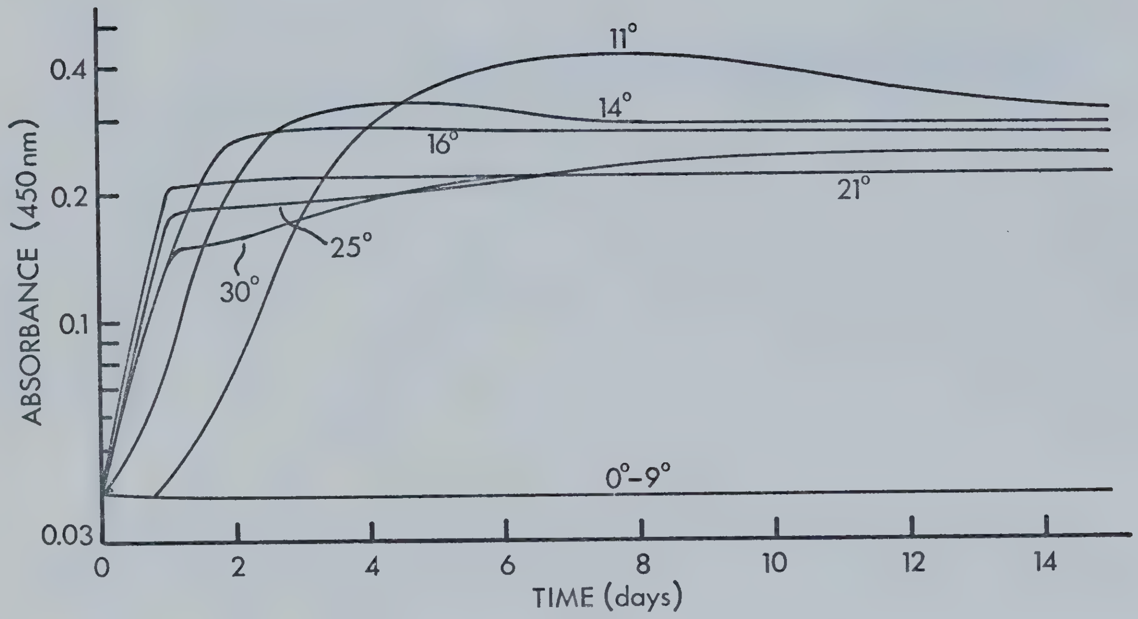

FIG. 20. Effect of temperature on the growth of *Salmonella heidelberg* in glucose-salt broth at pH 5.

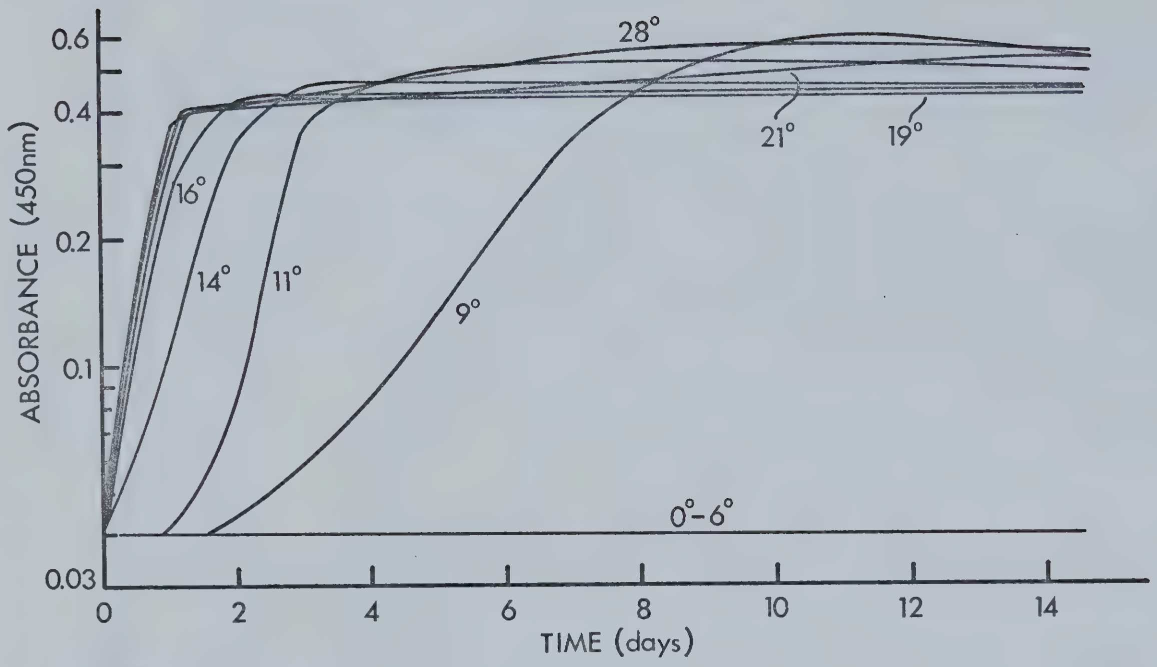

FIG. 21. Effect of temperature on the growth of *Salmonella heidelberg* in glucose-salt broth at pH 6.

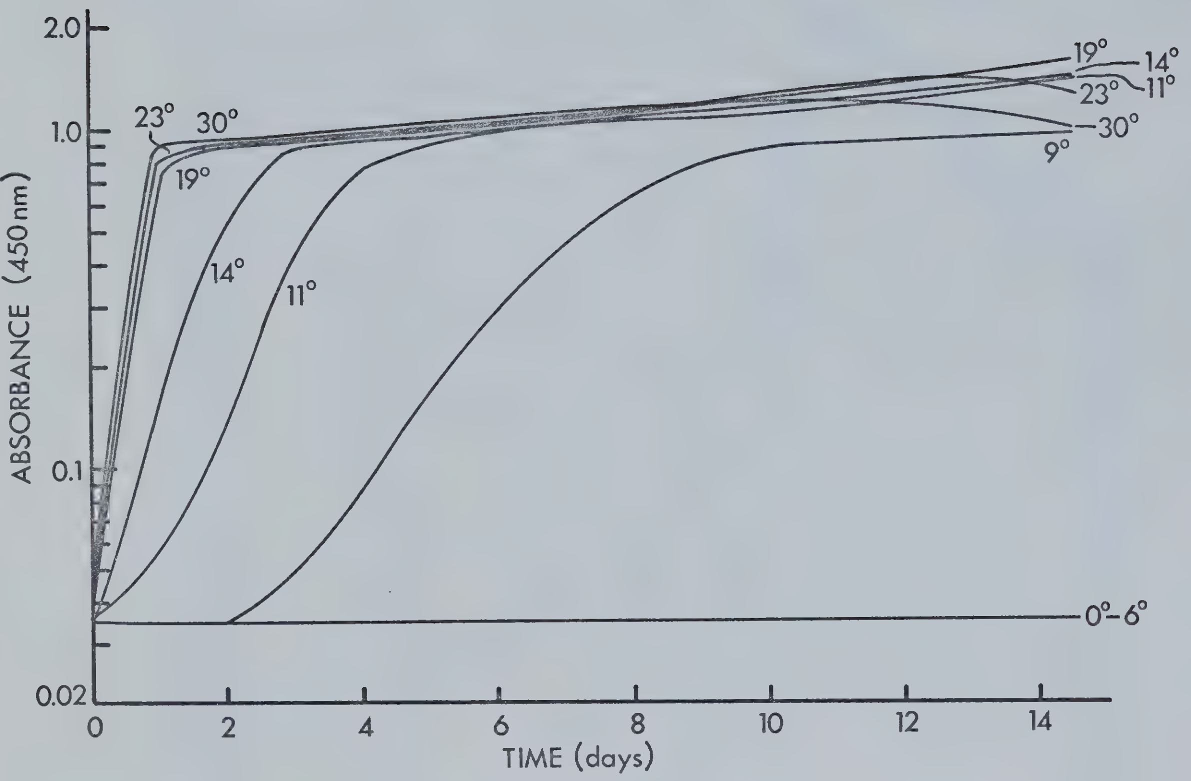

FIG. 22. Effect of temperature on the growth of *Salmonella heidelberg* in glucose-salt broth at pH 7.

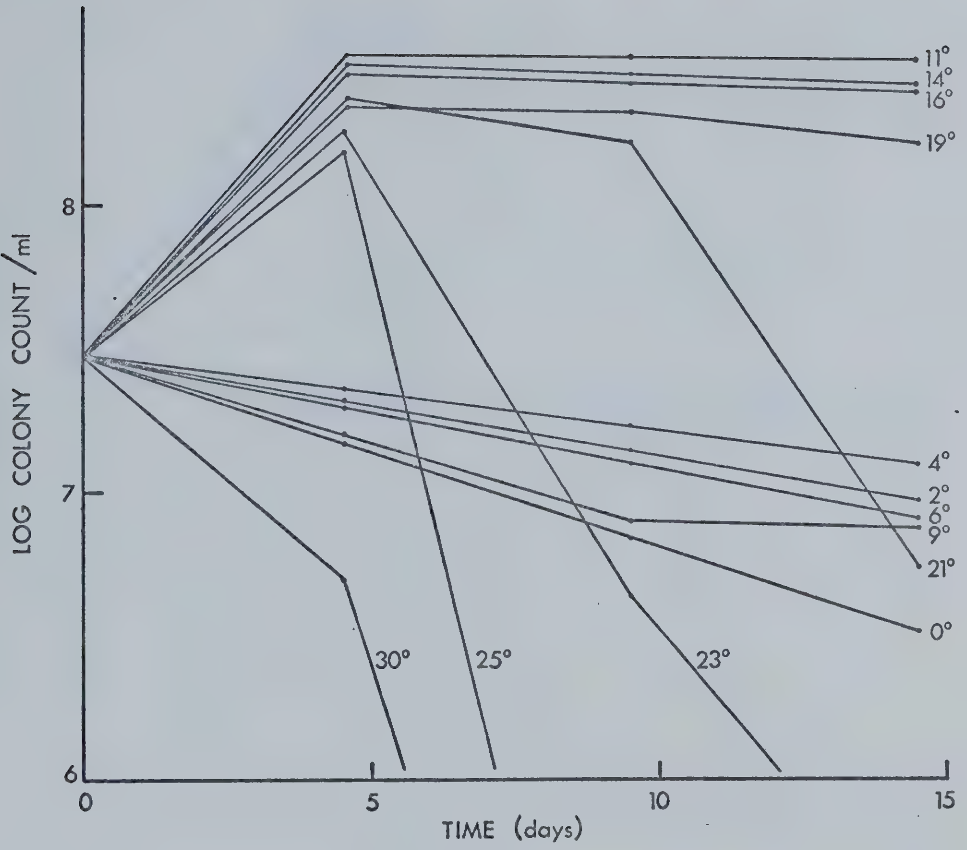

FIG. 23. Effect of temperature on the growth and viability of *Salmonella heidelberg* in glucose-salt broth at pH 5.

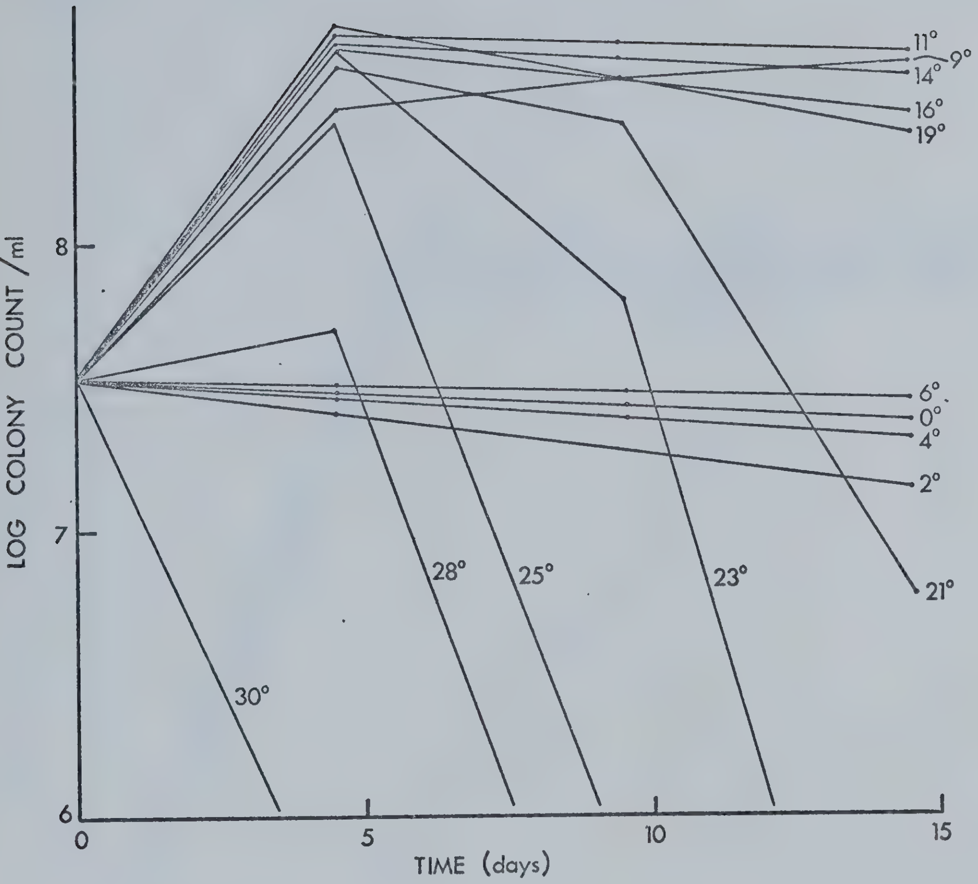

FIG. 24. Effect of temperature on the growth and viability of *Salmonella heidelberg* in glucose-salt broth at pH 6.

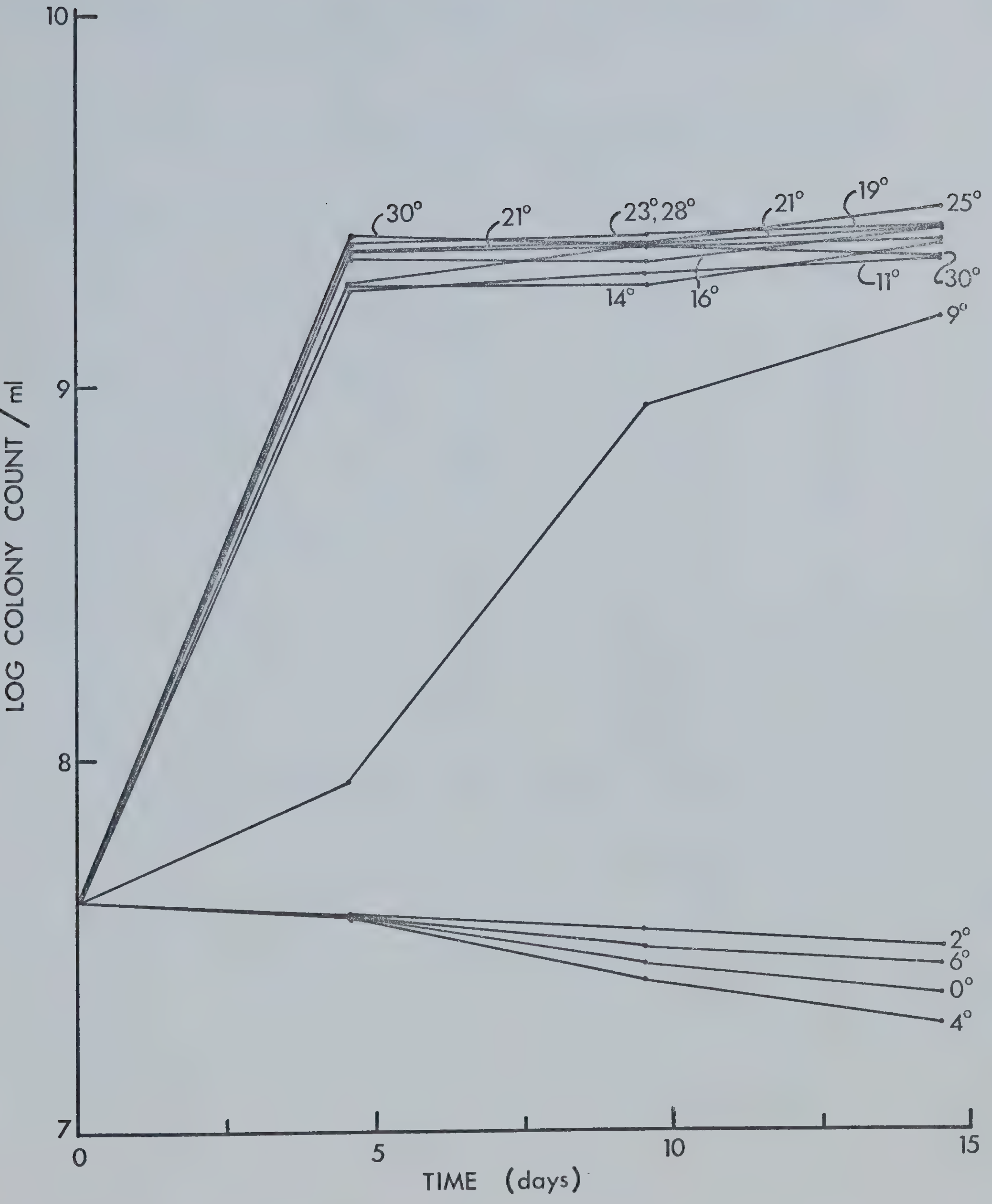

FIG. 25. Effect of temperature on the growth and viability of *Salmonella heidelberg* in glucose-salt broth at pH 7.

TABLE 4. Final pH of cultures of *Salmonella heidelberg* after growth at different temperatures in glucose-salt broth, pH 5, 6 and 7.

	Final pH after growth at an initial pH of		
Temperature	5	6	7
0	5.0	6.0	7.0
2	5.0	6.0	7.0
4	*n.d.*	6.0	7.0
6	5.0	6.0	7.0
9	5.0	4.8	6.7
11	4.3	4.9	6.8
14	4.4	4.9	6.8
16	4.4	4.9	6.9
19	4.4	4.9	7.0
21	4.4	4.7	7.0
23	4.3	4.6	7.1
25	4.2	4.6	7.1
28	4.2	4.6	7.1
30	4.2	4.6	7.1

n.d. - not done.

inoculated into GSB pre-tempered at the test temperature. Growth was immediate and exponential at 18° and at higher temperatures (Figs. 26, 27 and 28) but at 15° or lower temperatures growth was preceded by a lag period (Figs. 28 and 29). The lag period at 15, 9 and 7° could be as long as 2, 8 and 12 hr respectively. The pattern of ATP synthesis was very similar to that of growth (Figs. 26, 27, 28 and 29). Immediately upon temperature shift to 15° or lower, there was a sharp decrease in ATP synthesis in the lag phase. However, synthesis was resumed just before the initiation of growth. Even at 7 and 9°, temperatures close to the minimal growth temperature of the organism, appreciable amounts of ATP were synthesized corresponding very closely to the growth rate. At 7°, only trace amounts of ATP were excreted from the cells into the medium (Fig. 29). Since in this growth medium, GSB, growth was limited by the energy source, there was a drop in ATP content when the culture entered the stationary phase of growth. The rapidity of the drop in ATP content was affected by the growth temperature. The higher the growth temperature, the more rapid was the decrease in ATP content at the stationary phase.

The change in generation time with growth temperature in GSB is presented in Fig. 30. The generation time of *S. heidelberg* at 37° was 60 min and this time increased progressively with decreasing temperature until at 18° it was 196 min. At temperatures lower than 18°, the generation time increased rapidly. The generation time at 15, 9 and 7° were 342, 1320 and 1980 min respectively. A typical relationship between growth rate and incubation temperature, plotted according to Arrhenius is shown in Fig. 31. As the growth temperature is decreased from 37 to 18°, the growth rate decreases progressively and shows a

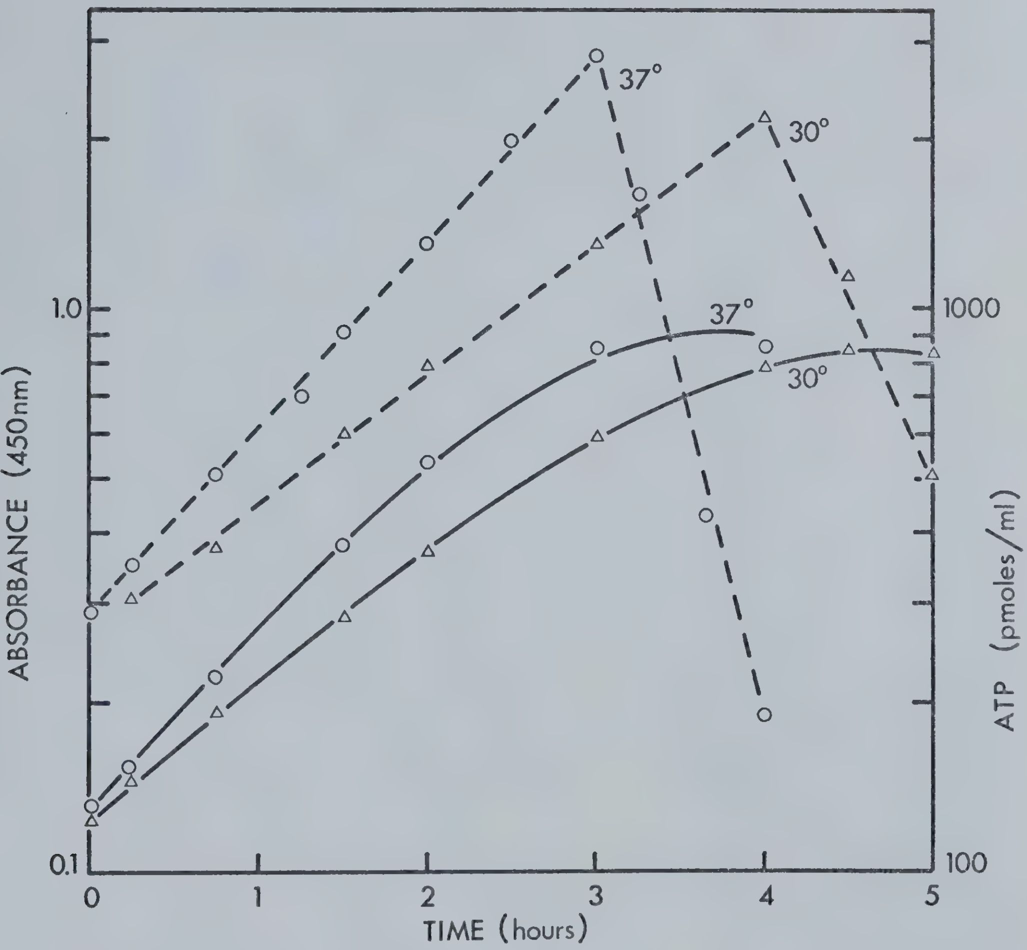

FIG. 26. Effect of temperature on growth and ATP production of *Salmonella heidelberg* in glucose-salt broth.

Symbols: ————————, absorbance;

-----------, ATP.

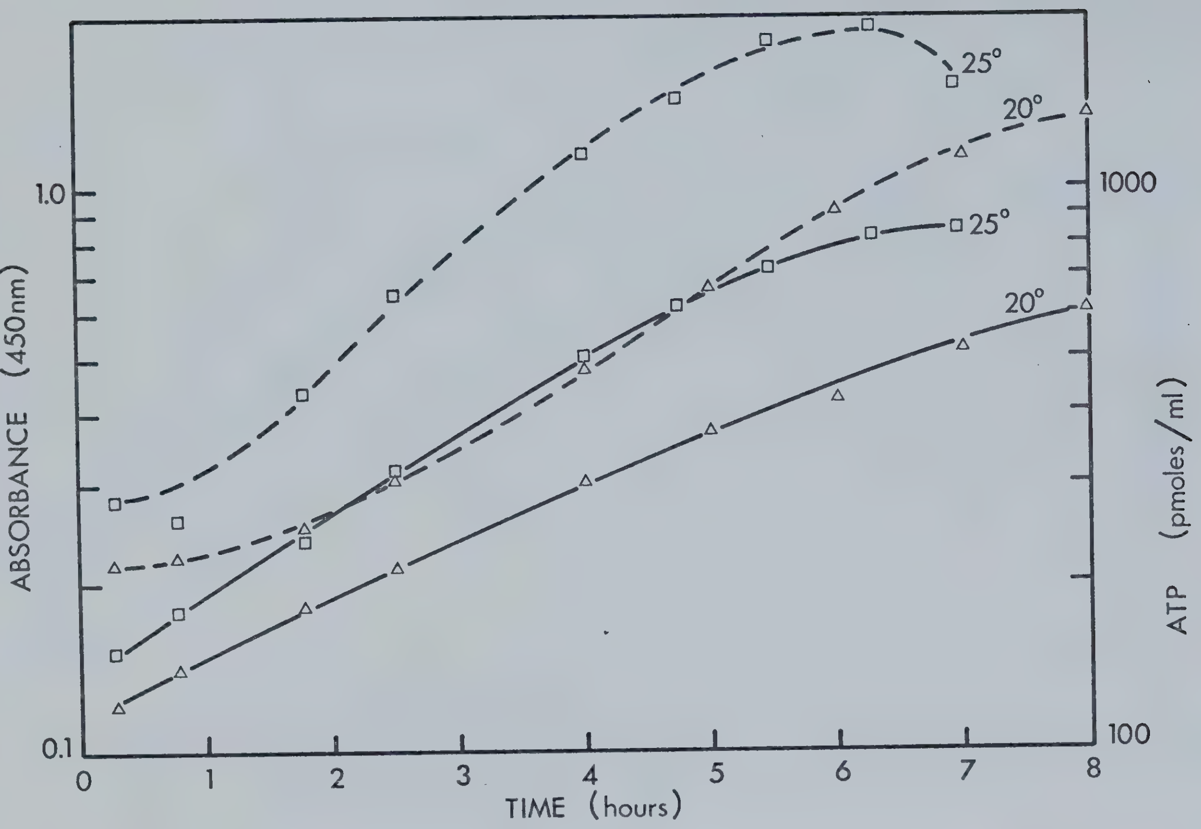

FIG. 27. Effect of temperature on growth and ATP production of *Salmonella heidelberg* in glucose-salt broth.

Symbols: ———, absorbance; -----, ATP.

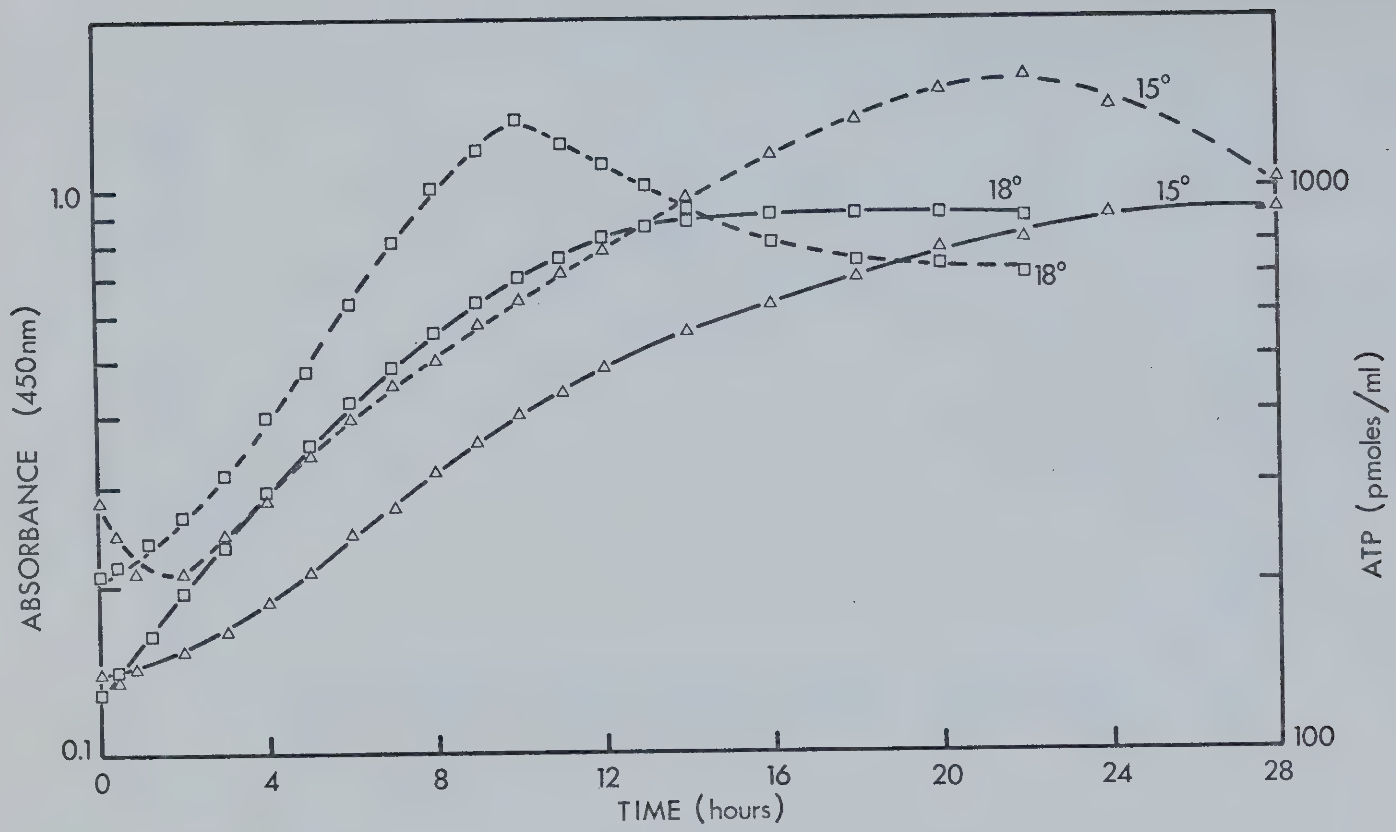

FIG. 28. Effect of temperature on growth and ATP production of *Salmonella heidelberg* in glucose-salt broth.

Symbols: ———, absorbance; -----, ATP.

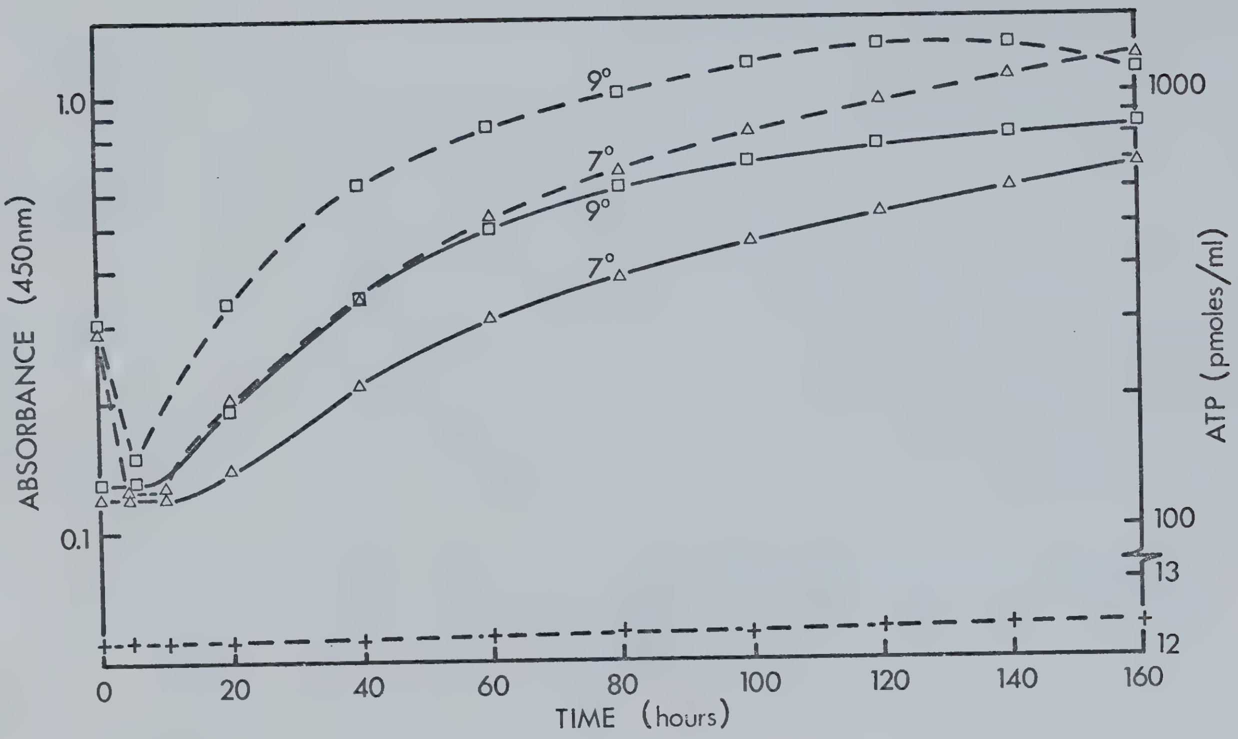

FIG. 29. Effect of temperature on growth and ATP production of *Salmonella heidelberg* in glucose-salt broth.

Symbols: ———, absorbance; -----, ATP; --+--, ATP leaked from cells incubated at 7°.

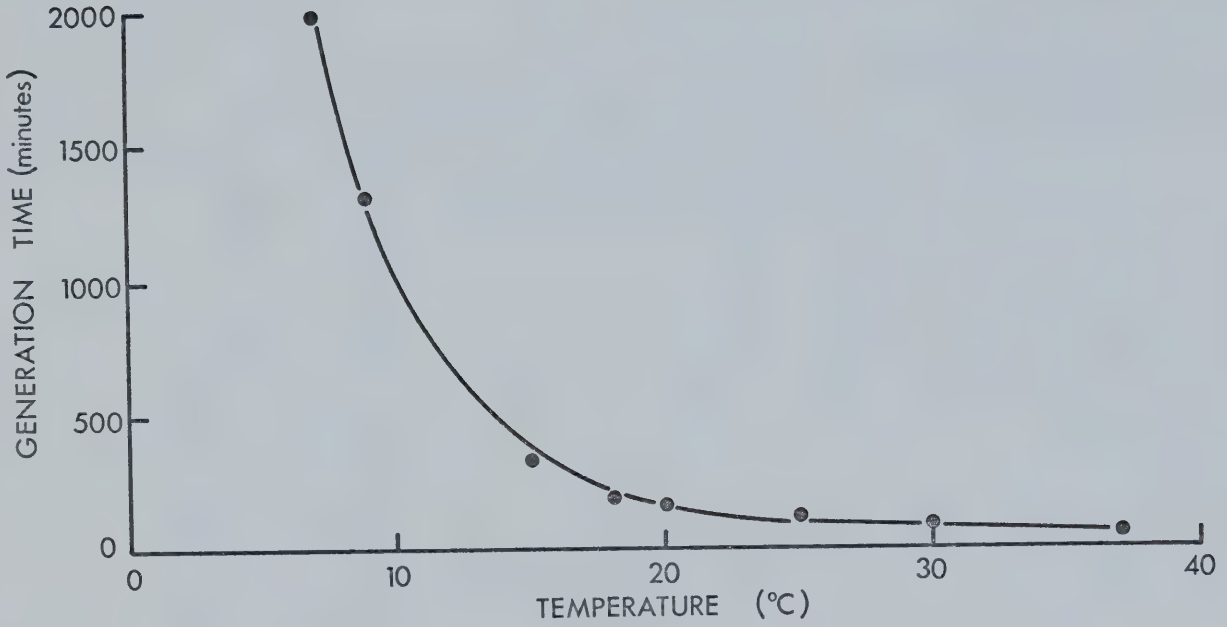

FIG. 30. Effect of temperature on the generation time of *Salmonella heidelberg* grown in glucose-salt broth.

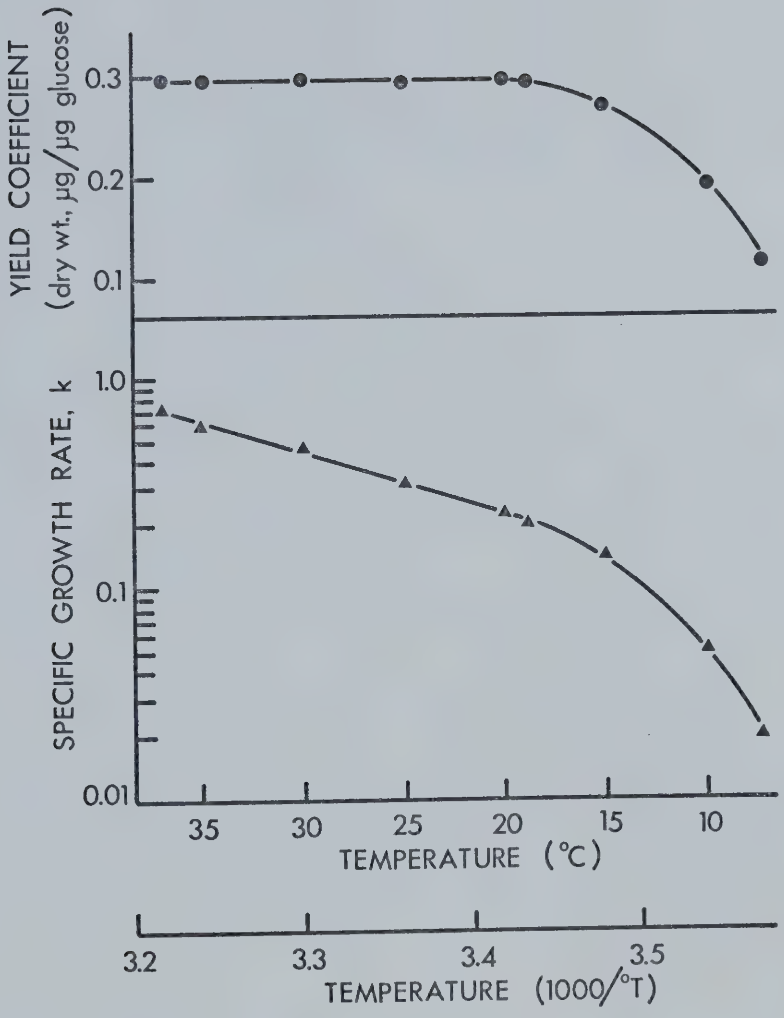

FIG. 31. Effect of temperature on specific growth rate and yield coefficient of *Salmonella heidelberg* in glucose-salt broth.

linear relationship between the logarithm of growth rate and the reciprocal of the absolute temperature. At about 15°, the curve deviates from linear. The linearity of the curve is further distorted at 9 and 7° with greatly lowered specific growth rates. The deviation from linearity in low temperature growth is reflected in decreased cell yield (Fig. 31). Cell yield was relatively constant from 37 to 18° below which the yield was no longer independent of growth rate and began to fall off precipitously.

Growth of the organism did not occur at 5° in glucose-salt broth (Fig. 32). Instead the culture showed progressive loss of the ability to form colonies. The loss of viability was much more apparent when the culture was plated on desoxycholate agar and MacConkey agar than on tryptic soy agar + 0.5% yeast extract (TSYA), glucose-salt agar and brilliant green agar. The difference in counts on these media, although negligible at the start, becomes progressively greater with time. This increased sensitivity to certain selective media is a well-known phenomenon of cellular injury and has been observed as a result of heating, freezing, freeze-drying, irradiation and chemical treatments. Since there is little information on the effects of non-freezing temperatures below the minimal growth temperature on microbial activity (Jackson, 1974), further experiments were carried out using *S. heidelberg* grown in glucose-salt broth or tryptic soy broth.

III. Cold Injury and Recovery of *Salmonella heidelberg*

A. GSB Grown Culture, Cold Stored in GSB

When exponential growing *S. heidelberg* in GSB was inoculated into fresh media pre-tempered at 0, 3, 5 and 6°, there was no growth from

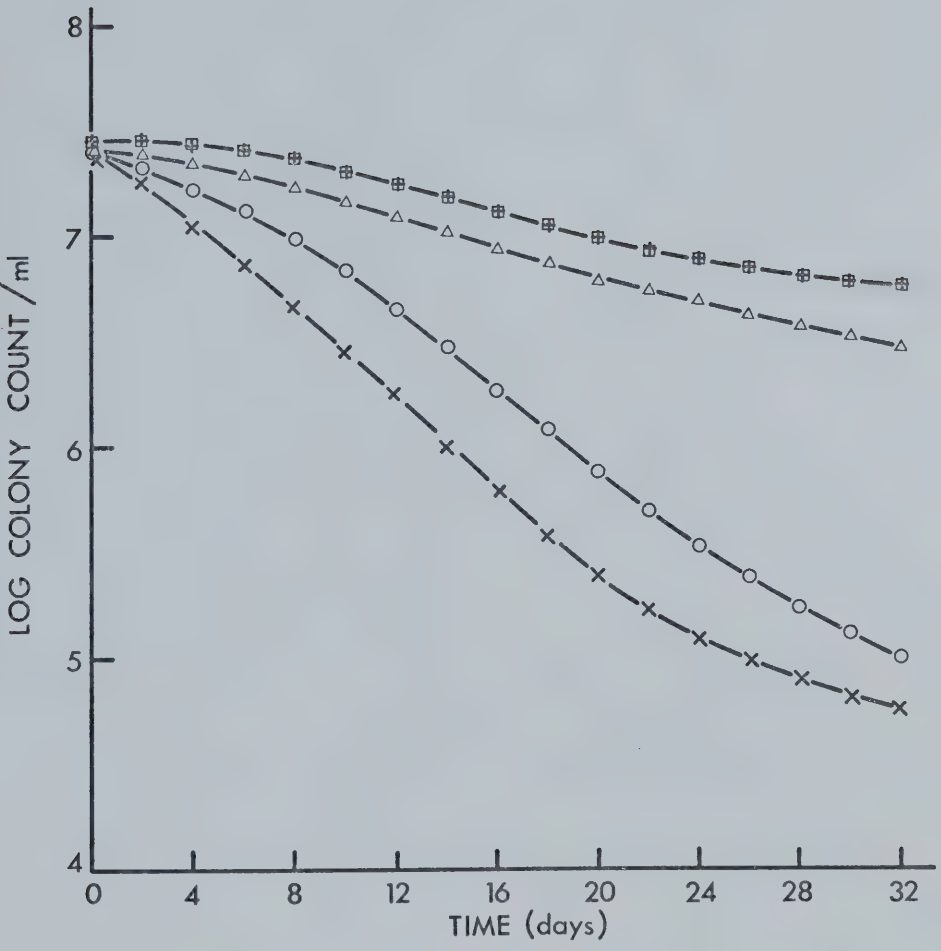

FIG. 32. Viability of *Salmonella heidelberg* in glucose-salt broth at 5°; inoculum grown in GSB at 37°.

Plating media: □, tryptic soy agar + 0.5% yeast extract; +, glucose-salt agar; Δ, brilliant green agar; o, desoxycholate agar; x, MacConkey agar.

0-5° and only limited growth at 6° during the 10 days' incubation as indicated by the counts on TSY agar (Fig. 33). The gradual decline in counts on TSY agar at 0° indicates that some of the cells died. As the storage temperature increased from 0°, death became less and less obvious. Although there was no drastic death of cells during storage, counts on the selective medium, desoxycholate agar decreased after 2 days' storage and became pronounced after 10 days' storage at 0, 3 and 5° resulting in a difference of 2, 1.3 and 0.9 log cycles between counts on the two media. Cultures stored at 6° also showed slight sensitivity to desoxycholate agar after 7 days of storage. The decrease in counts on desoxycholate agar compared to TSY agar was interpreted as loss of tolerance to the selective agar as a result of cell injury but not death, because the injured cells could actually recover from their sensitivity to desoxycholate agar in the absence of growth when incubated at 25° (Fig. 34). It can be seen that the cold-stored cells exhibit a lag of 2.5 hr in GSB at 25° when enumerated on TSY agar. During this lag phase, the cells completely recover their tolerance to the desoxycholate agar or MacConkey agar. Both the shape of the recovery curve and the absence of an increase in the counts on TSY agar during the lag or recovery period indicate that this is a recovery rather than a growth process. The minimal glucose-salt agar gave similar numbers of viable cells as TSY agar indicating that there was no increase in nutritional requirement of the cold-stored cells. This is further substantia~~lized~~ted by the fact that the injured cells had essentially the same rate of recovery in the complex rich medium, TSB and the minimal growth medium, GSB (Fig. 35).

To understand the mechanism involved during the recovery process,

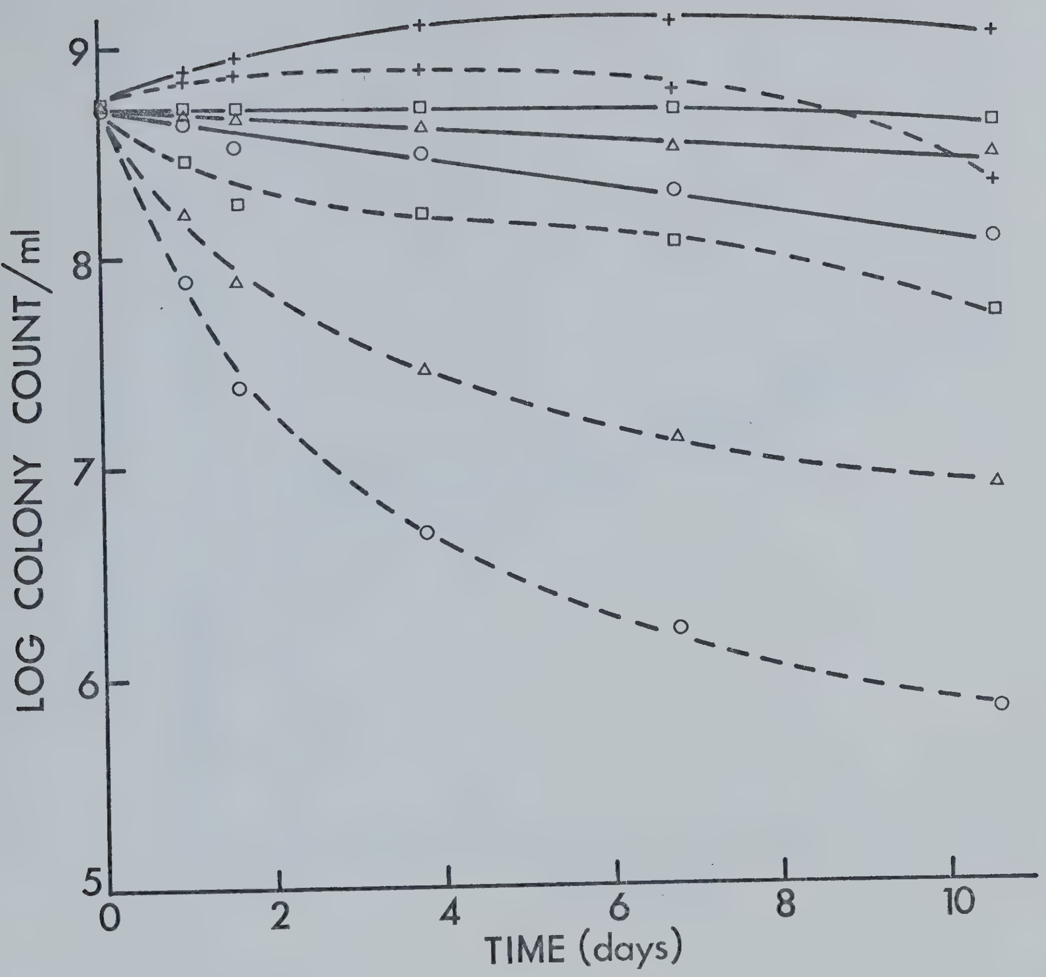

FIG. 33. Viability of *Salmonella heidelberg* in glucose-salt broth at low temperature; inoculum grown in GSB at 37°.

Temperature of incubation: o, 0°; Δ, 3°; □, 5°; +, 6°.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

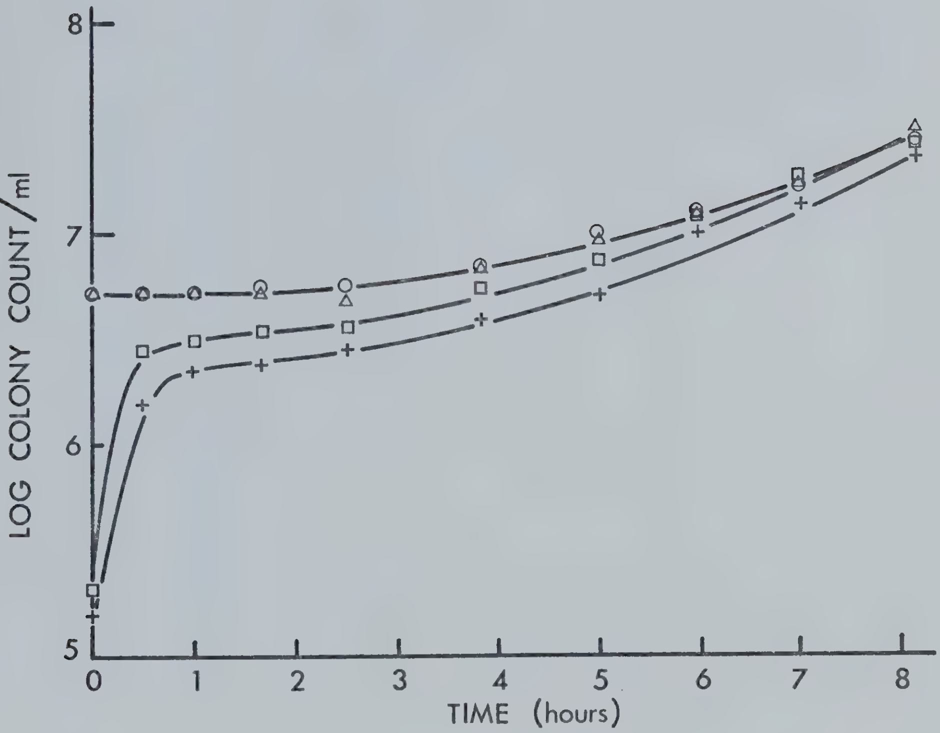

FIG. 34. Recovery and growth of *Salmonella heidelberg* in glucose-salt broth at 25°; organism grown in GSB at 37° and cold-injured for 4 days in GSB at 2°.

Plating media: o, tryptic soy agar + 0.5% yeast extract;
Δ, glucose-salt agar; □, desoxycholate agar;
+, MacConkey agar.

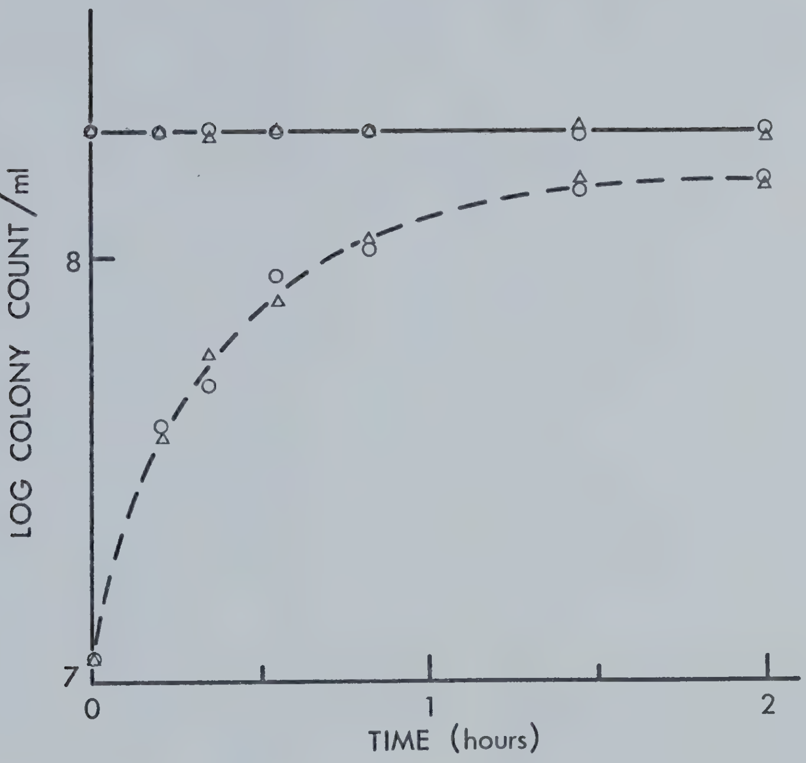

FIG. 35. Recovery of *Salmonella heidelberg* in glucose-salt broth and tryptic soy broth at 20°; organism grown in GSB at 37° and cold-injured for 4 days in GSB at 2°.

Recovery menstrua: o, GSB; △, TSB.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

various metabolic inhibitors were added to the recovery medium, GSB. Inhibitors employed were chloramphenicol, rifamycin, hydroxyurea, penicillin and 2,4-dinitrophenol, dissolved in GSB and then cooled to 0-2°. Portions of cold-injured cell culture were then added and the whole was incubated at 20°. To assess recovery, the number of colonies on desoxycholate agar at 0 and 2 hr were compared to the number of colonies on TSY agar. After 2 hr of incubation at 20°, the injured cells recovered their ability to grow on desoxycholate agar in the presence of all the inhibitors tested (Fig. 36). The level of cellular protein, RNA, DNA and ATP was also determined during recovery at 25° (Fig. 37). A great proportion of the injured cells had recovered their tolerance to desoxycholate agar within 40 min during which practically no increase in the levels of cellular protein, RNA and DNA was detected, whereas ATP levels increased rapidly.

The fact that injured cells can recover in the absence of protein, RNA, DNA and cell wall synthesis and do not show increased nutritional requirement indicates that the recovery process may be a simple process in which the endogenous reserve of the cells might play an important role. This was studied by suspending injured cells in phosphate buffer (0.063M, pH 7.2) and recovery was followed at 20°. As shown in Fig. 38, phosphate was able to support recovery. To further substantiate that the recovery process is a simple process without complex metabolic synthesis other inhibitors (hydroxyurea, penicillin, sodium cyanide, chloramphenicol or fluorouracil) were added to the simple recovery menstruum, phosphate buffer and the recovery process at 20° was followed for 2 hr (Fig. 39 and 40). The rate of recovery in the presence of all the inhibitors was very similar to that in phosphate

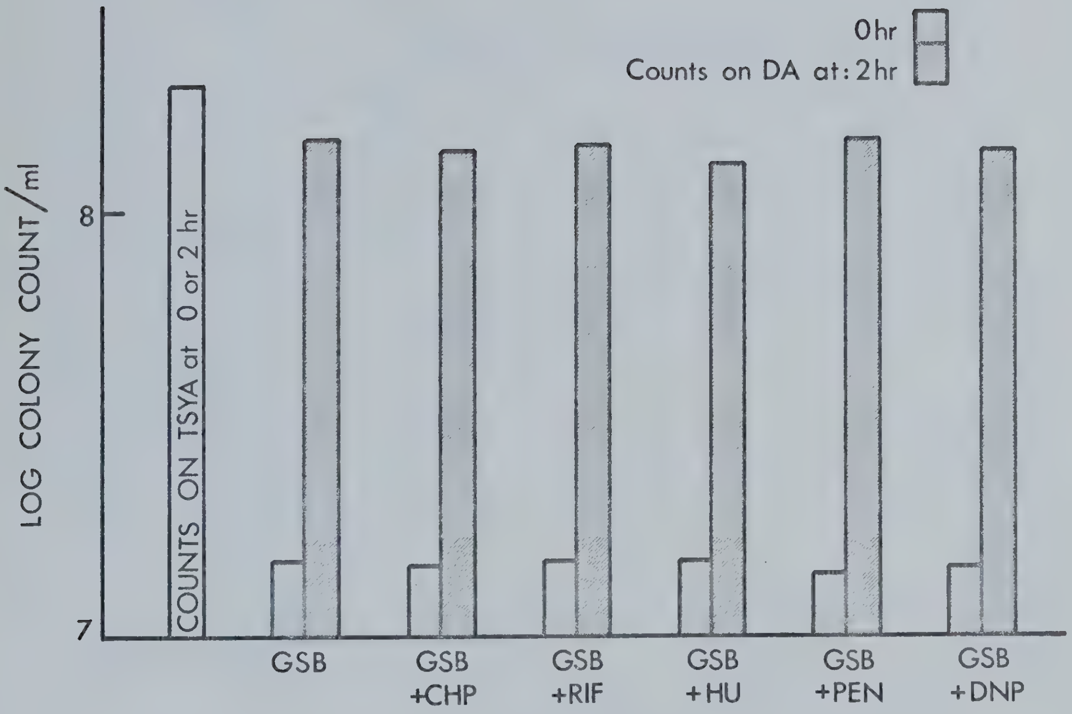

FIG. 36. Recovery of *Salmonella heidelberg* in glucose-salt broth plus different metabolic inhibitors at 20°; organism grown in GSB at 37° and cold-injured for 4 days in GSB at 2°. Inhibitors used were chloramphenicol (CHP), rifamycin (RIF), hydroxyurea (HU), penicillin (PEN) and 2,4-dinitrophenol (DNP). To indicate repair, the numbers on desoxycholate agar (DA) at 0 and 2 hr are presented and compared to that on tryptic soy agar + 0.5% yeast extract (TSYA).

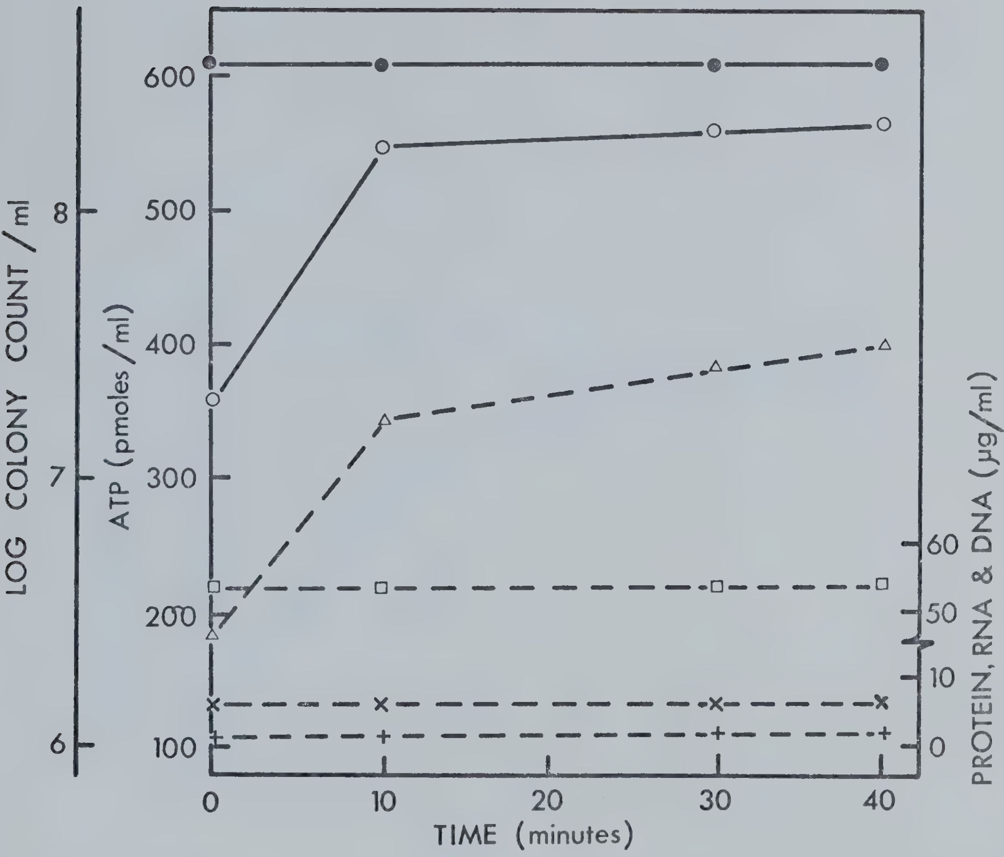

FIG. 37. Cellular level of ATP, protein, RNA and DNA of *Salmonella heidelberg* during recovery in glucose-salt broth at 25°; organism grown in GSB at 37° and cold-injured for 4 days in GSB at 2°.

Plating media: ●, tryptic soy agar + 0.5% yeast extract;
o, desoxycholate agar.

Cellular composition: Δ, ATP; □, protein; x, RNA; +, DNA.

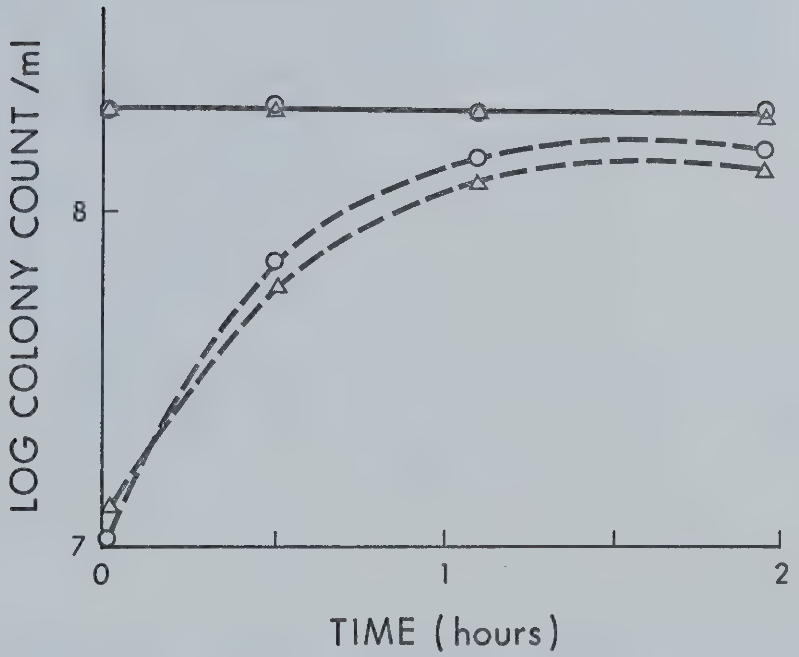

FIG. 38. Recovery of *Salmonella heidelberg* in glucose-salt broth and phosphate buffer at 20°; organism grown in GSB at 37° and cold-injured for 4 days in GSB at 2°.

Recovery menstrua: o, GSB; Δ, phosphate buffer.

Plating media: ———, tryptic soy agar + 0.5% yeast extract;
-----, desoxycholate agar.

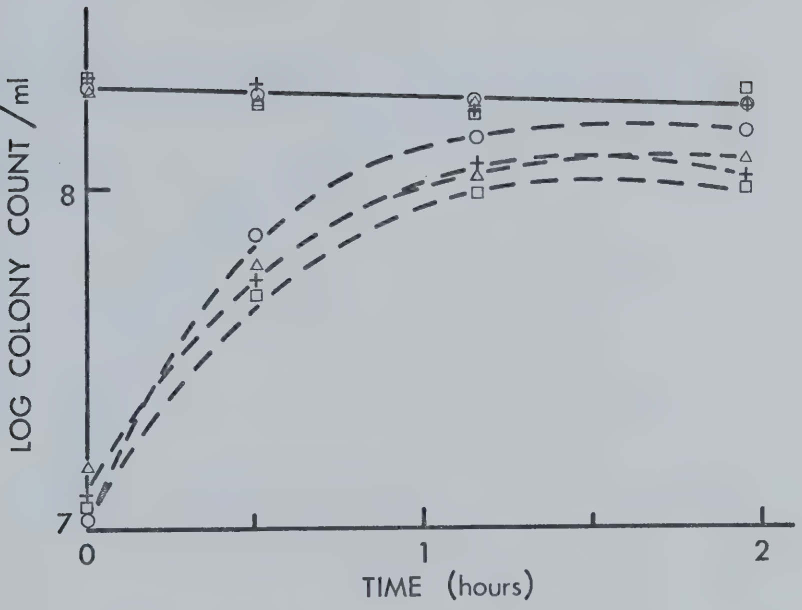

FIG. 39. Recovery of *Salmonella heidelberg* in phosphate buffer (P) plus hydroxyurea, penicillin or sodium cyanide at 20°; organism grown in GSB at 37° and cold-injured for 4 days in GSB at 2°.

Recovery menstrua: o, P; Δ, P + hydroxyurea; □, P + sodium cyanide; +, P + penicillin.

Plating media: ———, tryptic soy broth + 0.5% yeast extract; -----, desoxycholate agar.

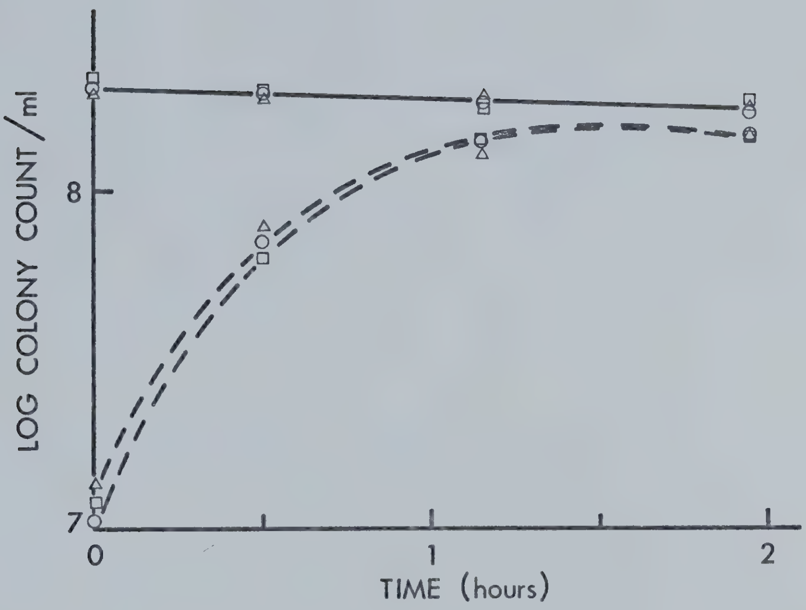

FIG. 40. Recovery of *Salmonella heidelberg* in phosphate buffer (P) plus chloramphenicol or fluorouracil at 20°; organism grown in GSB at 37° and cold-injured for 4 days in GSB at 2°.

Recovery menstrua: o, P; Δ, P + chloramphenicol; □, P + fluorouracil.

Plating media: ——, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

buffer alone and recovery was complete within 1.5 hr.

Further information on the behavior of cultures during storage in GSB at 2° was found with the observation that the addition of 2,4-dinitrophenol to the storage medium greatly protected the cells from increased sensitivity to desoxycholate agar (Fig. 41). After 4 days' storage, the viable counts, with and without 2,4-dinitrophenol, were 1.2×10^8 cells/ml and 7×10^6 cells/ml respectively. When sodium azide was added instead, the tolerance of the cells toward desoxycholate was protected. However, sodium azide also increased the mortality rate of the cells as indicated by a drop of 0.4 log cycle of counts on TSY agar.

2,4-dinitrophenol whether added a few minutes before or after the temperature shift to 2° protected the cells against leakage of cellular ATP during storage (Fig. 42). In its absence, the initial rate of ATP leakage was rapid and reached maximum at about 2 days' storage. The RNA and DNA content of the cultures during storage is shown in Fig. 43. The pattern of RNA and DNA degradation of the cells did not differ much whether stored in the presence or absence of 2,4-dinitrophenol, although the RNA level of the cells preincubated with the inhibitor for a few min before transfer to 2° was comparatively lower. It appears that RNA and DNA degradation bear no direct relationship to desoxycholate sensitivity of the injured cells.

During cold storage, a considerable amount of 260 nm absorbing material appeared in the storage medium (Fig. 44). Neither cell lysis nor death occurred as indicated by the absorbance of culture (Fig. 44) and counts on TSY agar (Fig. 41). In order to discover whether the cell membrane was damaged during cold storage, the ability of the dye, ANS to

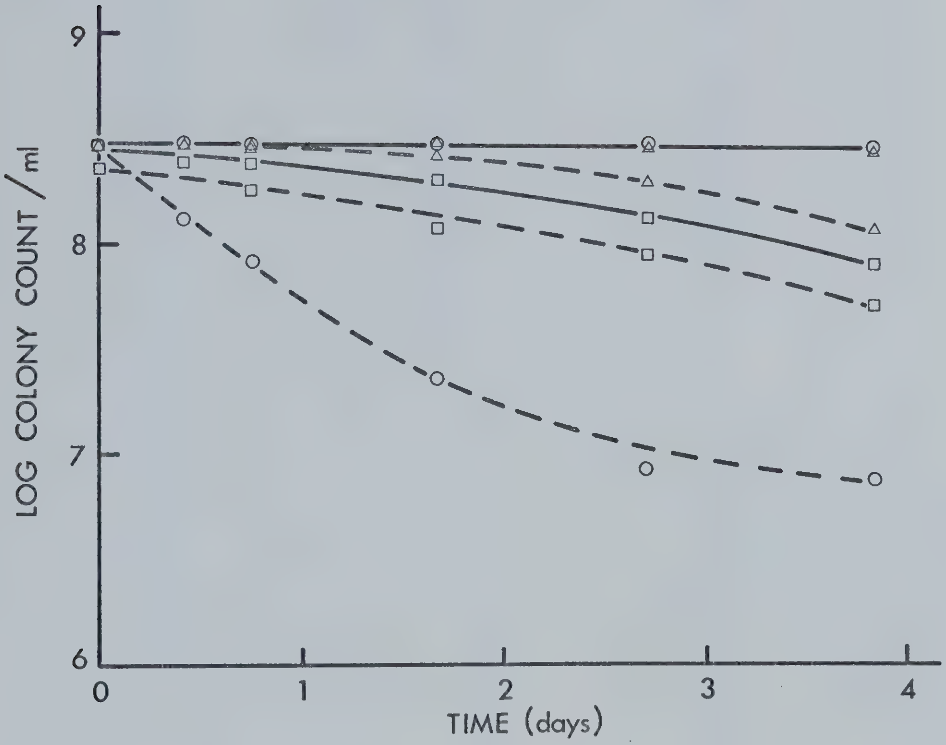

FIG. 41. Viability of *Salmonella heidelberg* in glucose-salt broth with or without inhibitors of ATP synthesis at 2°; inoculum grown in GSB at 37°.

Storage menstrua: o, GSB; Δ, GSB + 2,4-dinitrophenol; □, GSB + sodium azide.

Plating media: ——, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

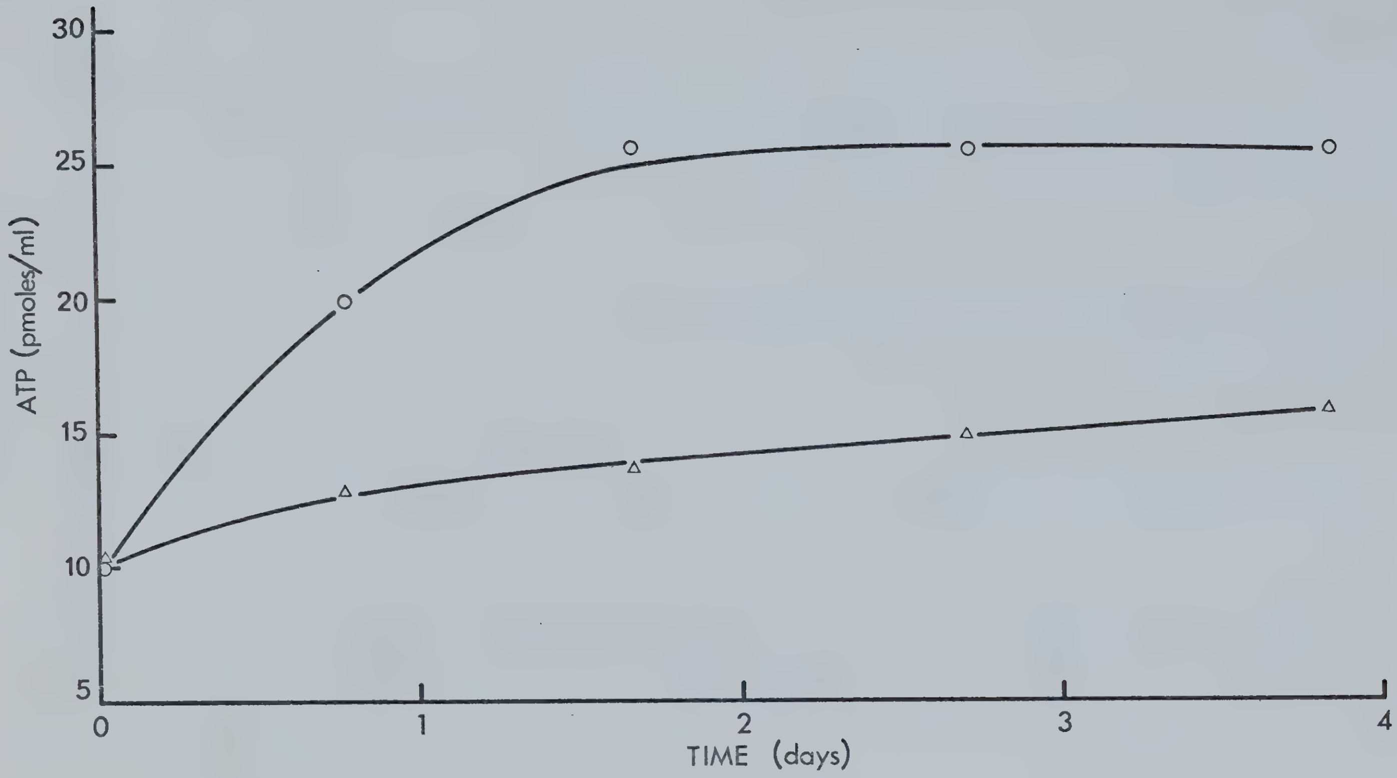

FIG. 42. Leakage of ATP from *Salmonella heidelberg* during storage at 2° in glucose-salt broth; inoculum grown in GSB at 37°.

Storage menstrua: O, GSB; Δ, GSB + 2,4-dinitrophenol.

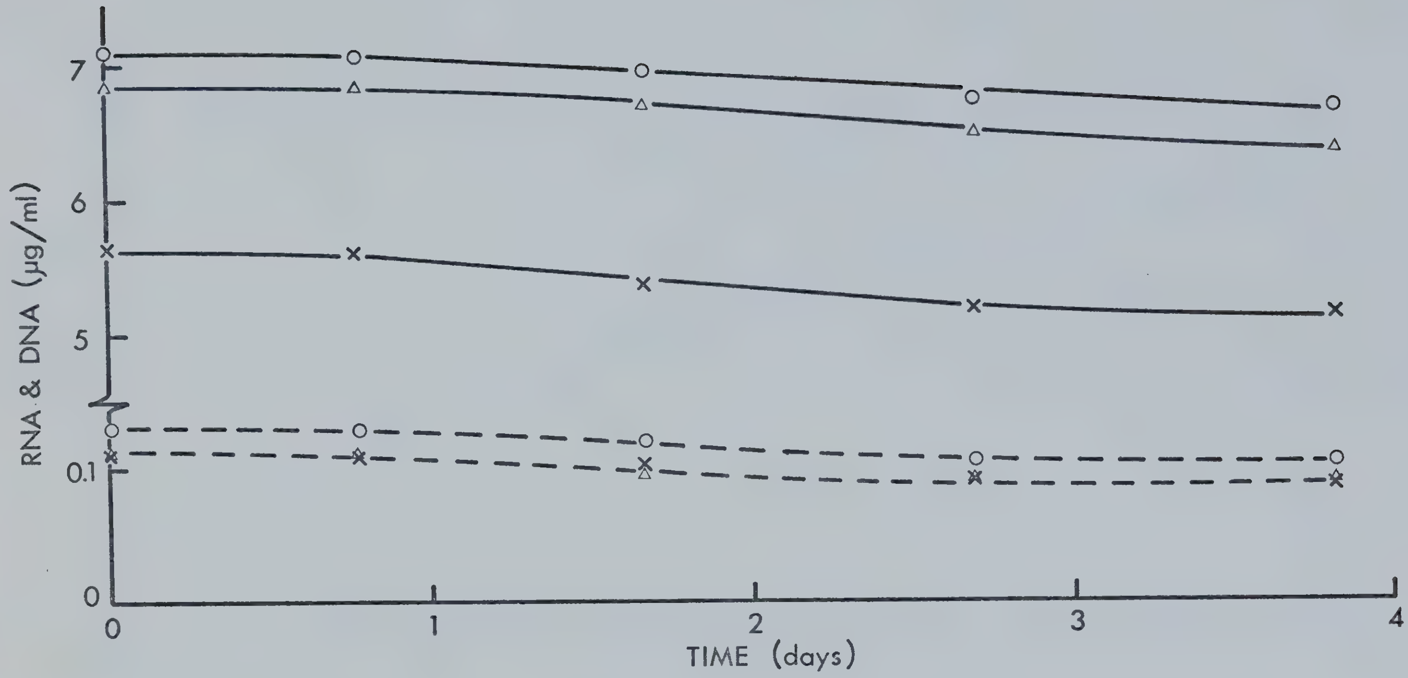

FIG. 43. RNA and DNA content of *Salmonella heidelberg* during storage at 2° in glucose-salt broth with or without inhibitors of ATP synthesis; inoculum grown in GSB at 37°.

Symbols: ———, RNA; -----, DNA; o, stored in GSB; Δ, stored in GSB + 2,4-dinitrophenol; x, GSB + 2,4-dinitrophenol with which the inoculum had been incubated for 15 min at 37° before temperature shift.

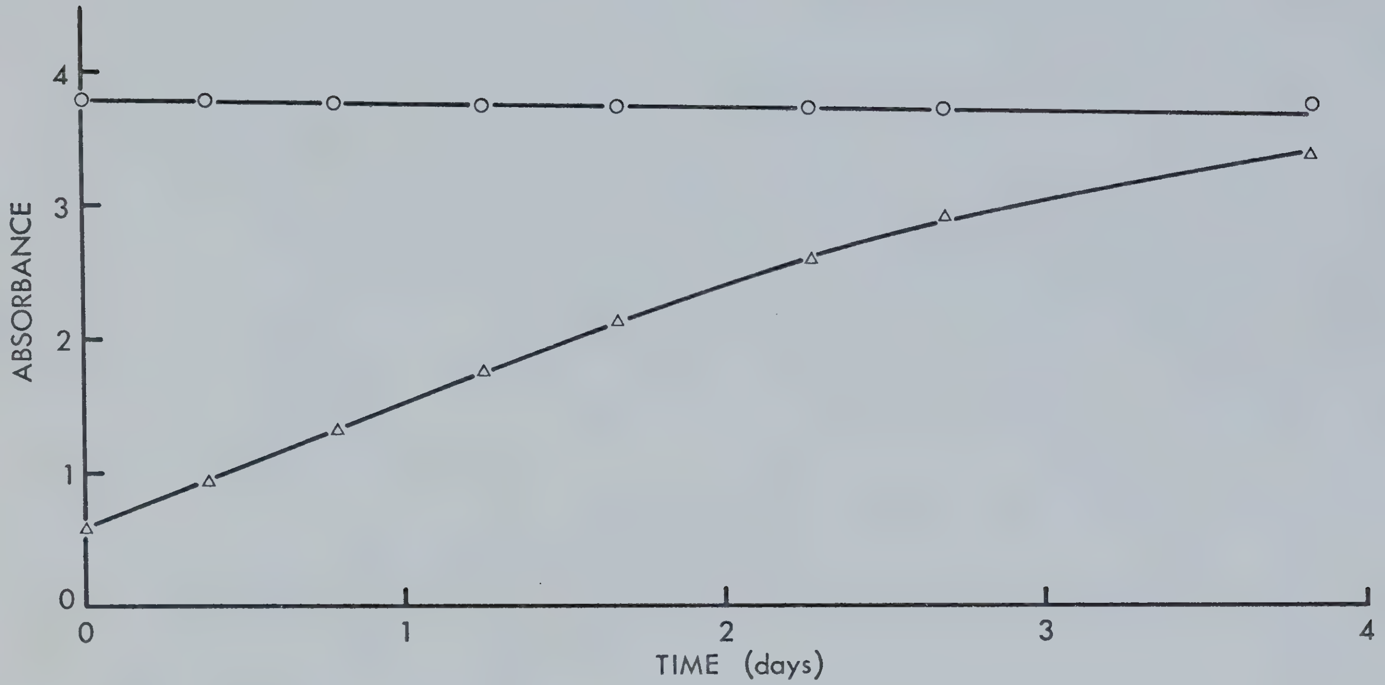

FIG. 44. Leakage of U.V. absorbing material from *Salmonella heidelberg* during storage at 2° in glucose-salt broth; inoculum grown in GSB at 37°.

Symbols: o, absorbance of culture at 450 nm; Δ, absorbance of culture supernatant at 260 nm.

penetrate the cells was determined (Fig. 45). After storage, ANS was able to enter into the injured cells and they fluoresced strongly compared to the normal cells. When the injured culture was incubated at 37°, it recovered its impermeability to ANS.

Previous experiments showed that *S. heidelberg* was rendered sensitive to desoxycholate agar with reference to TSY agar during cold storage. Another phenomenon was observed when *S. heidelberg* was inoculated into fresh GSB pre-tempered at 5°, no matter whether the inoculum was obtained directly from the growth vessel at 37° or by harvesting the culture at 18° and then resuspending in small portions of GSB at room temperature. Immediately upon transferring the culture to 5°, the viable count on the rich medium, TSY agar, was considerably less (2.5 log cycles) than the count on the minimal glucose-salt agar (Fig. 46). On further incubation at 5°, the culture gradually recovered from its sensitivity to TSY agar. The recovery process was almost complete after 8 hr at 5° and after 2 hr at 20°. On the other hand, the count on desoxycholate agar was not significantly different from that on glucose-salt agar during the 8 hr incubation at 5° (data not shown). The failure of *S. heidelberg* to grow on TSY agar upon chilling is analogous to the well documented phenomenon of 'cold shock' (Rose, 1968), but it would be more appropriate to interpret the present observation as damage rather than death of the cells as the organisms can actually recover the ability to grow on TSY agar even at 5°.

The fact that the recovery of *S. heidelberg* from its sensitivity to TSY agar can occur at 5° indicates that certain metabolic activities can proceed at such a low temperature and contribute to the recovery process. As some activities continue at these temperatures and others

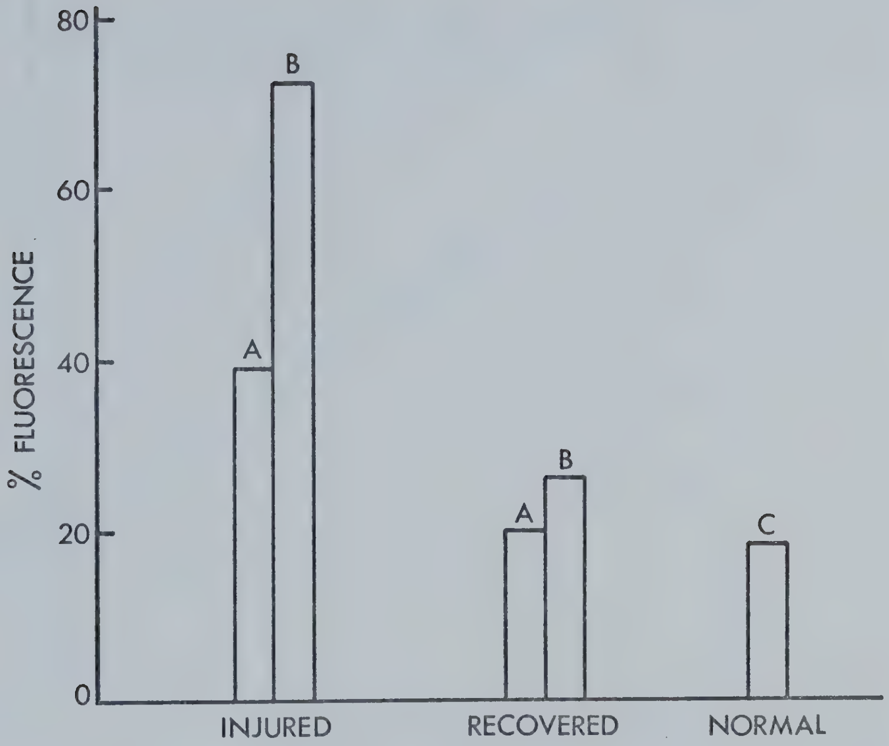

FIG. 45. The ability of ANS to penetrate cold-injured and recovered cells of *Salmonella heidelberg*. Culture A was injured in glucose-salt broth for 4 days at 2° and recovered for 20 min at 37°; culture B was similarly injured for 10 days and recovered for 1 hr; culture C was normal cells without cold storage.

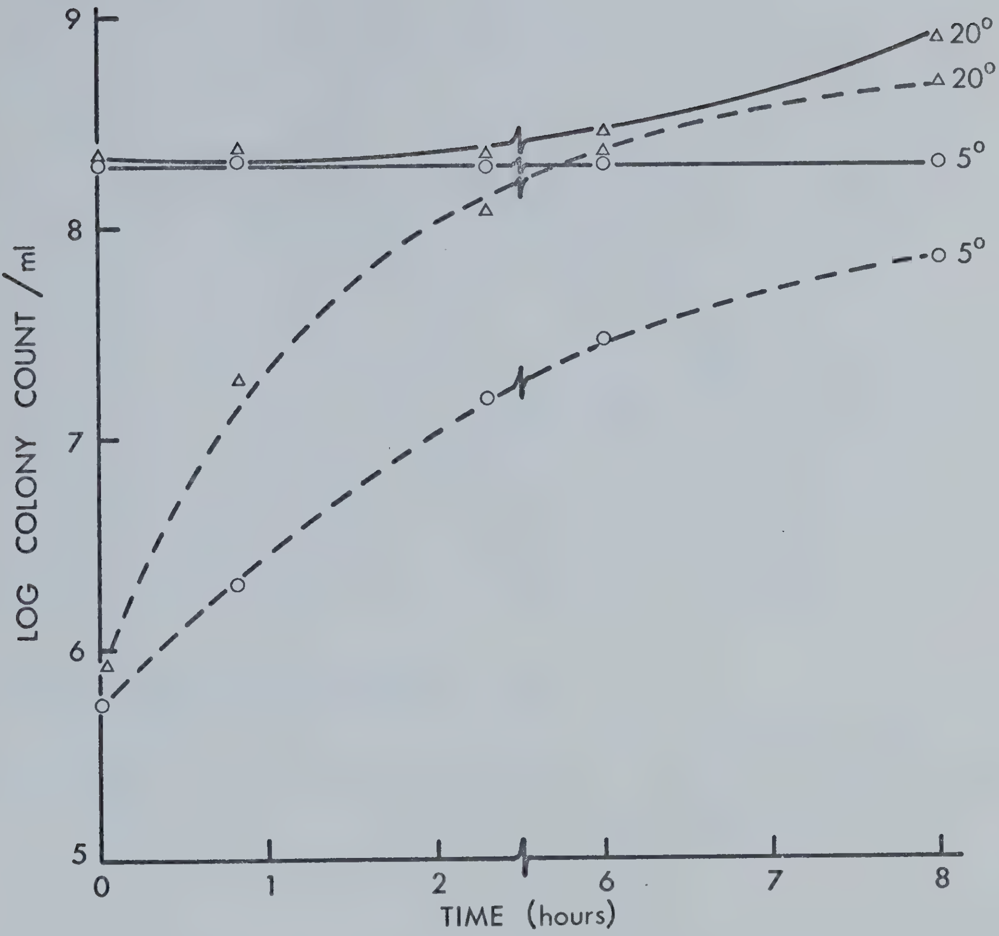

FIG. 46. Loss of the ability of *Salmonella heidelberg* to grow on tryptic soy agar + 0.5% yeast extract upon transfer from 37 to 5° and its recovery in glucose-salt broth at 5 and 20°; inoculum grown in GSB at 37°.

Plating media: ———, glucose-salt agar; -----, tryptic soy agar + 0.5% yeast extract.

do not, it was considered to be of interest to find out which activities were involved in the recovery of *S. heidelberg* from its sensitivity to TSY agar. This was studied by incorporating metabolic inhibitors into GSB and following recovery at 5° (Fig. 47, 48, and 49). Of the inhibitors tested, chloramphenicol, hydroxyurea, penicillin and 2,4-dinitrophenol did not show a significant amount of inhibition either on the rate or total amount of recovery in GSB during the 8 hr incubation at 5°. However, both rifamycin and rifampin inhibited the recovery process completely. Both inhibitors stop RNA synthesis by irreversibly binding to DNA-dependent RNA polymerase. Rifampin also produced some cell death during the recovery period (Fig. 49).

B. TSB Grown Culture, Cold Stored in TSB

Exponential cultures of *S. heidelberg* grown in TSB at 37° were transferred to fresh medium pre-tempered at 5, 6 and 8°. Viability was determined by plating out the cultures at regular intervals on TSY agar and desoxycholate agar. The results are shown in Fig. 50. It can be seen that the loss of viability was greater on desoxycholate agar than on TSY agar at 5 and 6°. The difference in counts on the two media, although negligible at the start, became progressively greater with time. Cultures incubated at 8° gave similar counts on both media and growth was demonstrated at this temperature. Some growth also occurred at 6°. At 5°, the culture was injured more rapidly and to a greater extent than that at 6°.

The effect of temperature on recovery was studied by suspending the injured cells in TSB pre-tempered at 5 to 35° (Fig. 51). The data show that the recovery process is temperature dependent. The higher the temperature, the faster is the rate of recovery. The recovery at

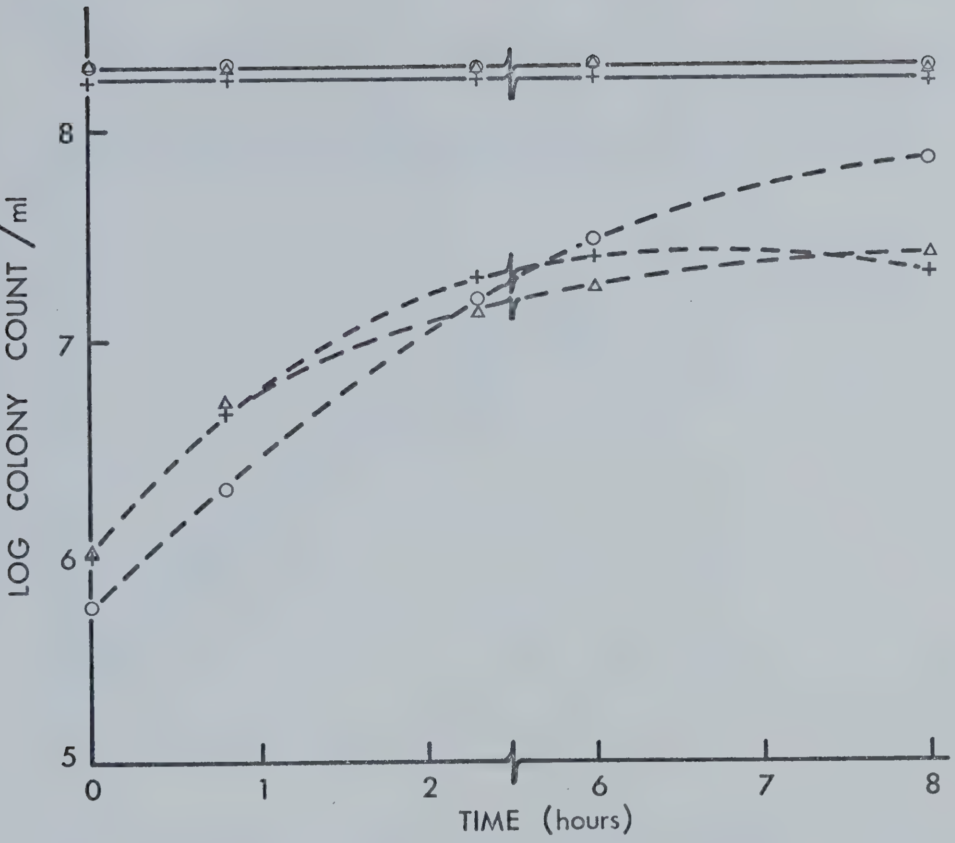

FIG. 47. Recovery of the ability of *Salmonella heidelberg* to grow on tryptic soy agar + 0.5% yeast extract in glucose-salt broth plus chloramphenicol or hydroxyurea at 5°; organism grown in GSB at 37° and cold-shocked at 5°.

Recovery menstrua: o, GSB; Δ, GSB + chloramphenicol; +, GSB + hydroxyurea.

Plating media: ———, glucose-salt agar; -----, tryptic soy agar + 0.5% yeast extract.

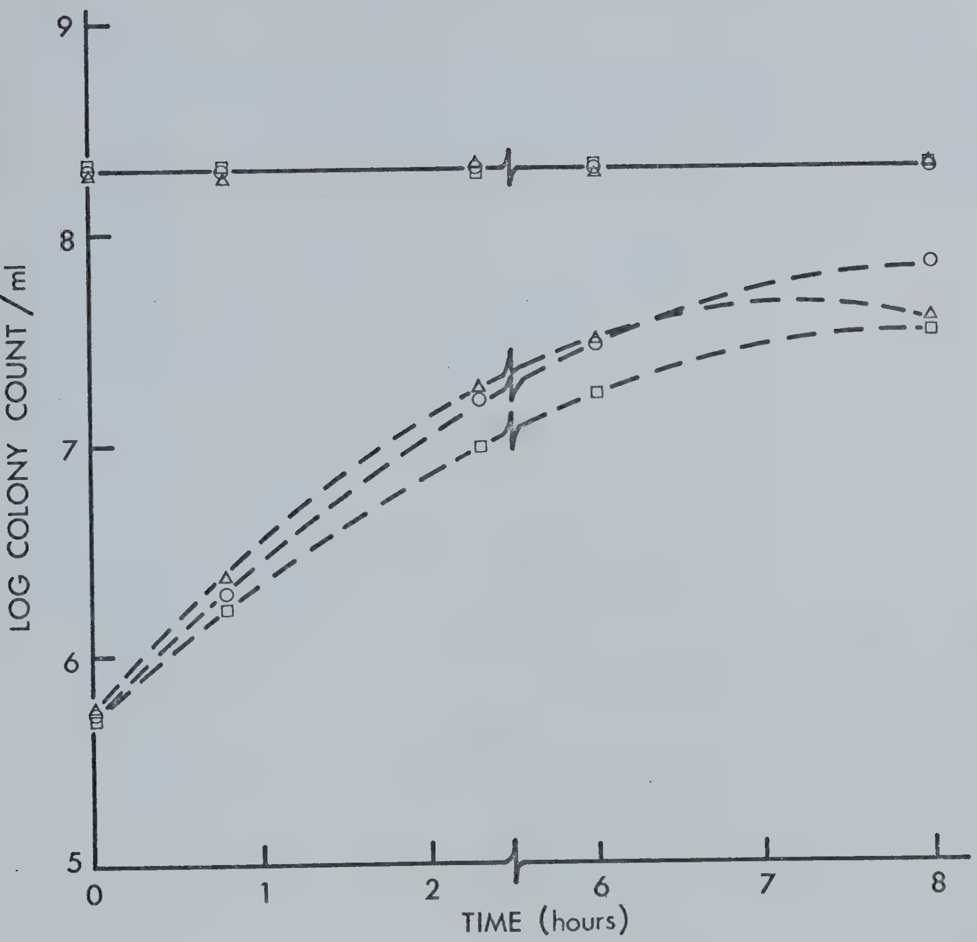

FIG. 48. Recovery of the ability of *Salmonella heidelberg* to grow on tryptic soy agar + 0.5% yeast extract in glucose-salt broth plus penicillin or 2,4-dinitrophenol at 5°; organism grown in GSB at 37° and cold-shocked at 5°.

Recovery menstrua: o, GSB; Δ, GSB + penicillin; □, GSB + 2,4-dinitrophenol.

Plating media: ———, glucose-salt agar; -----, tryptic soy agar + 0.5% yeast extract.

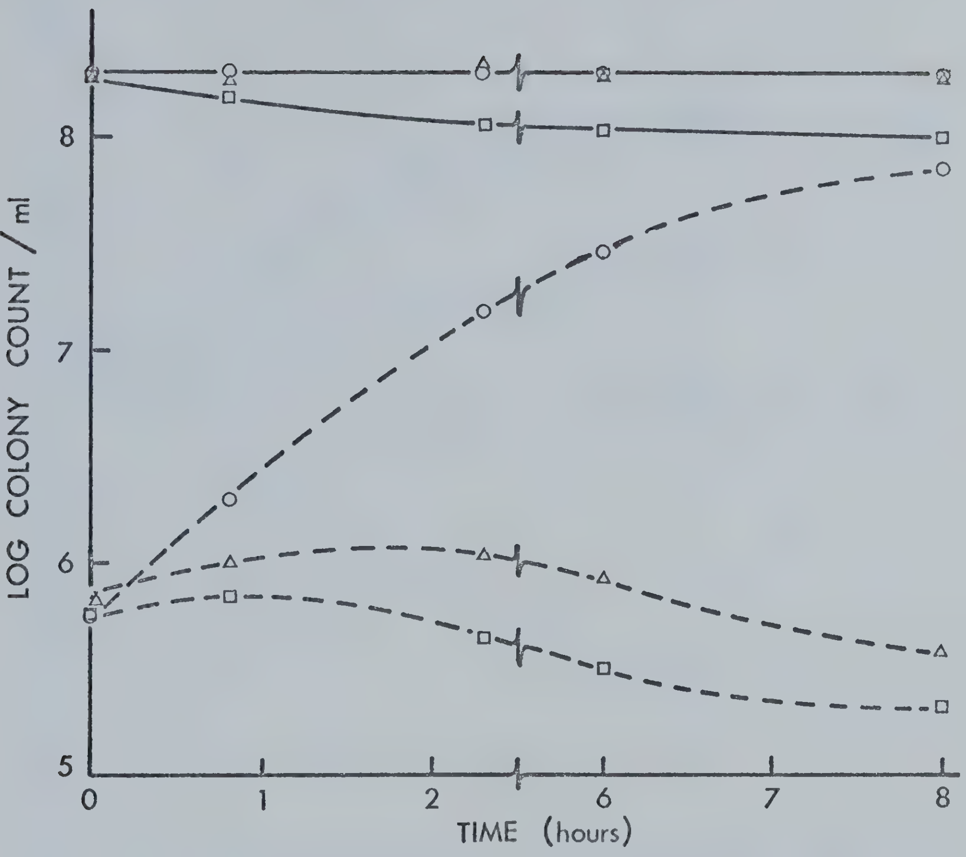

FIG. 49. Recovery of the ability of *Salmonella heidelberg* to grow on tryptic soy agar + 0.5% yeast extract in glucose-salt broth plus rifamycin and rifampin at 5°; organism grown in GSB at 37° and cold-shocked at 5°.

Recovery menstrua: o, GSB; Δ, GSB + rifamycin; □, GSB + rifampin.

Plating media: ———, glucose-salt agar; -----, tryptic soy agar + 0.5% yeast extract.

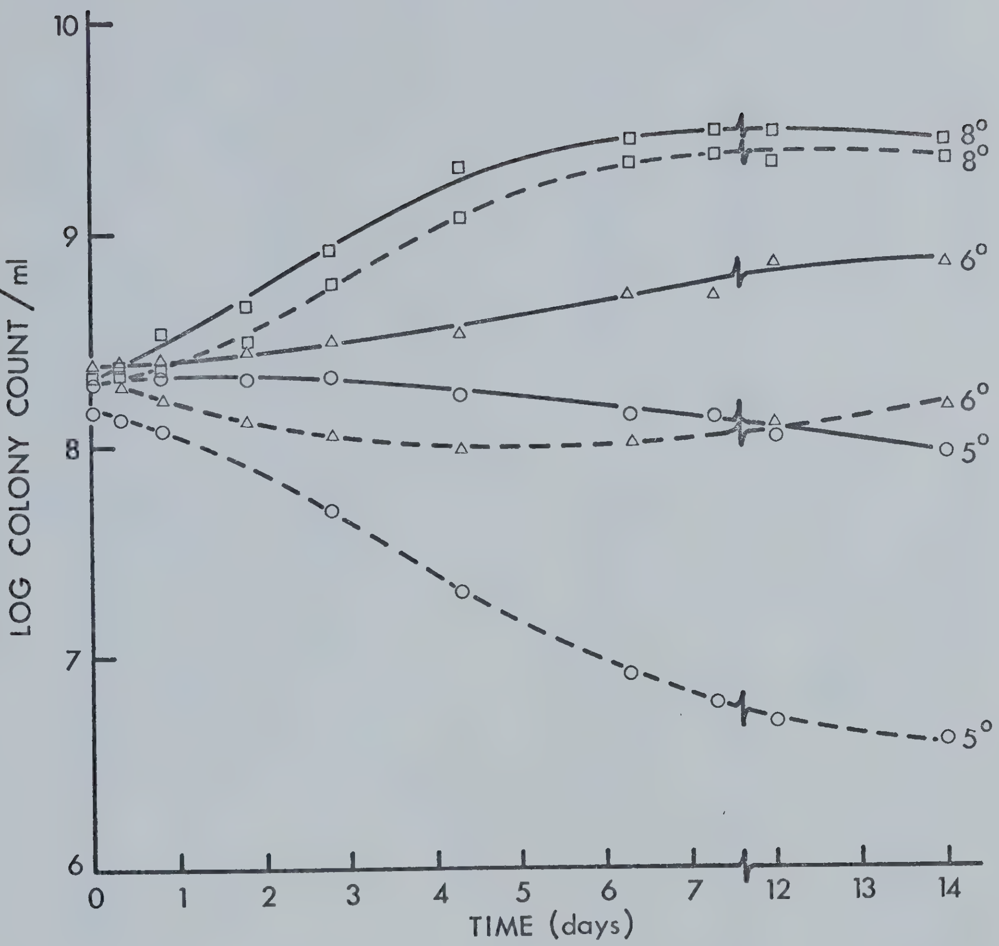

FIG. 50. Growth and viability of *Salmonella heidelberg* in tryptic soy broth at 5, 6 and 8°; inoculum grown in tryptic soy broth at 37°.

Plating media: ——, tryptic soy agar + 0.5% yeast extract;
-----, desoxycholate agar.

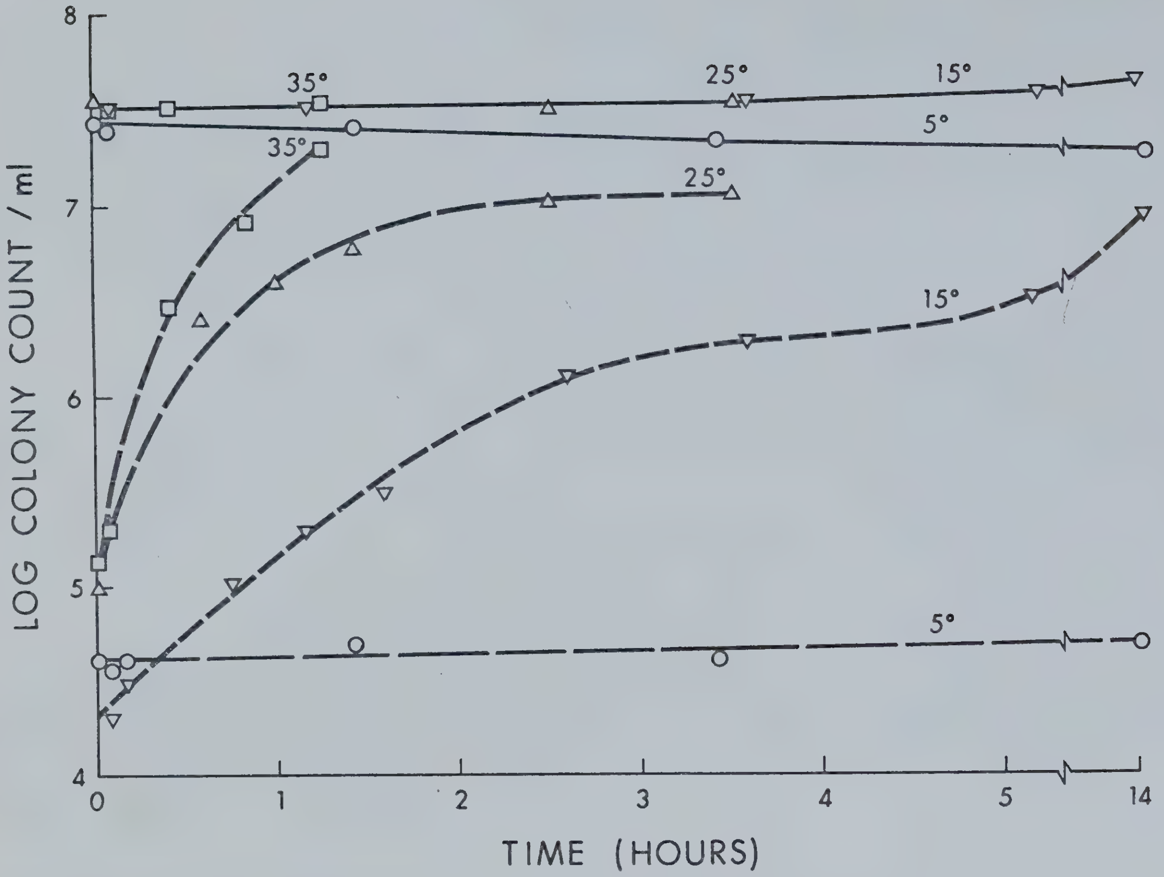

FIG. 51. Recovery of *Salmonella heidelberg* in tryptic soy broth at different temperatures; organism grown in TSB at 37° and cold-injured for 6 days in TSB at 2°.

Plating media: ———, tryptic soy agar + 5% yeast extract; -----, desoxycholate agar.

25 and 35° was very rapid while that at 15° was much slower. There was no recovery at 5° even after 14 hr incubation.

Recovery was also evaluated at 7 pH levels from 4 to 10. The increase in numbers of colonies on desoxycholate agar, indicating the rate and extent of recovery, was rapid and essentially the same at pH 6, 7 and 8, but much less at pH 5 and 9 (Figs. 52 and 53). Numbers observed on TSY agar indicating death dropped gradually at pH 5 and 9. There was no recovery at pH 4 and 10 but rather rapid death was observed (data not shown).

The effect of metabolic inhibitors on the recovery process is shown in Figs. 54-57. None of the following inhibitors affected the recovery significantly: chloramphenicol, nalidixic acid, rifampin, sodium azide, gramicidin, 2,4-dinitrophenol, penicillin and cycloserine. The recovery was essentially complete at 2 hr incubation in TSB at 20°. The ability of injured cells to recover in simple recovery menstrua was then tested with glucose-salt broth, phosphate buffer (0.063M, pH 7.2) and distilled water at 20° (Fig. 58). Recovery in phosphate buffer was almost equally efficient as in glucose-salt broth. Distilled water also permitted significant amount of recovery.

So far, it has been demonstrated that injury of *S. heidelberg* occurred at refrigeration temperatures. As some microbial activities can continue at these temperatures and others cannot, it was again decided to determine the effect of specific metabolic inactivation on cell injury and death. This experiment was conducted with *S. heidelberg* grown in TSB at 37° and transferred to fresh medium plus one of the following inhibitors pre-tempered at 2°: 2,4-dinitrophenol, rifampin, hydroxyurea, penicillin, sodium azide, oligomycin, chloramphenicol and

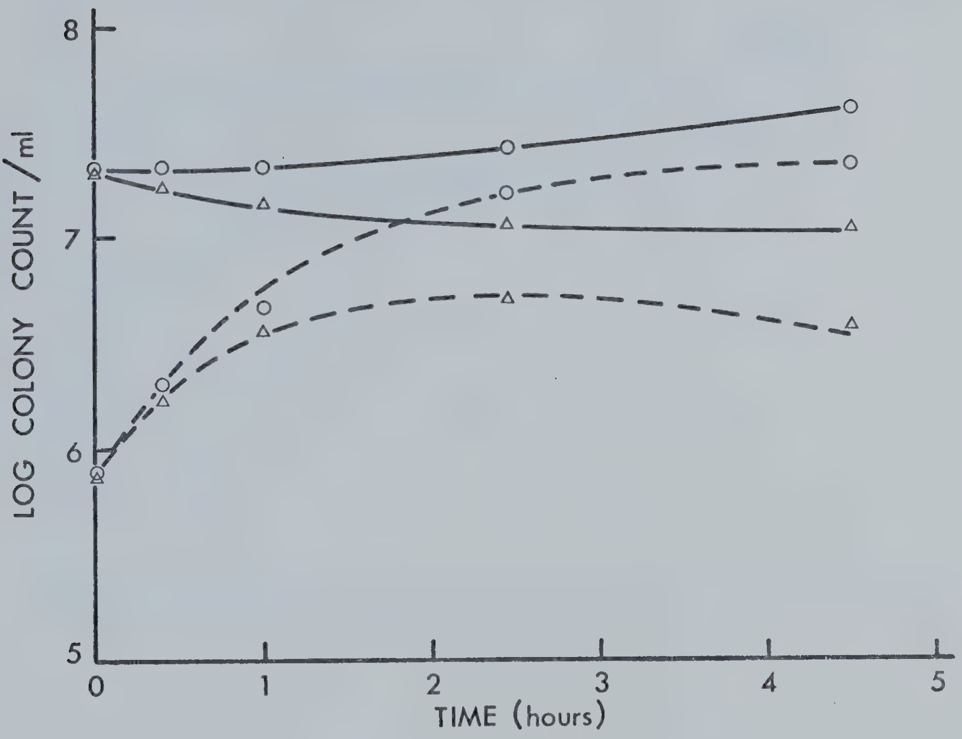

FIG. 52. Recovery of *Salmonella heidelberg* in tryptic soy broth of pH 5 and 6 at 20°; organism grown in TSB at 37° and cold-injured for 4 days in TSB at 2°.

pH of broth: Δ, 5; o, 6.

Plating media: ———, tryptic soy agar + 0.5% yeast extract;
-----, desoxycholate agar.

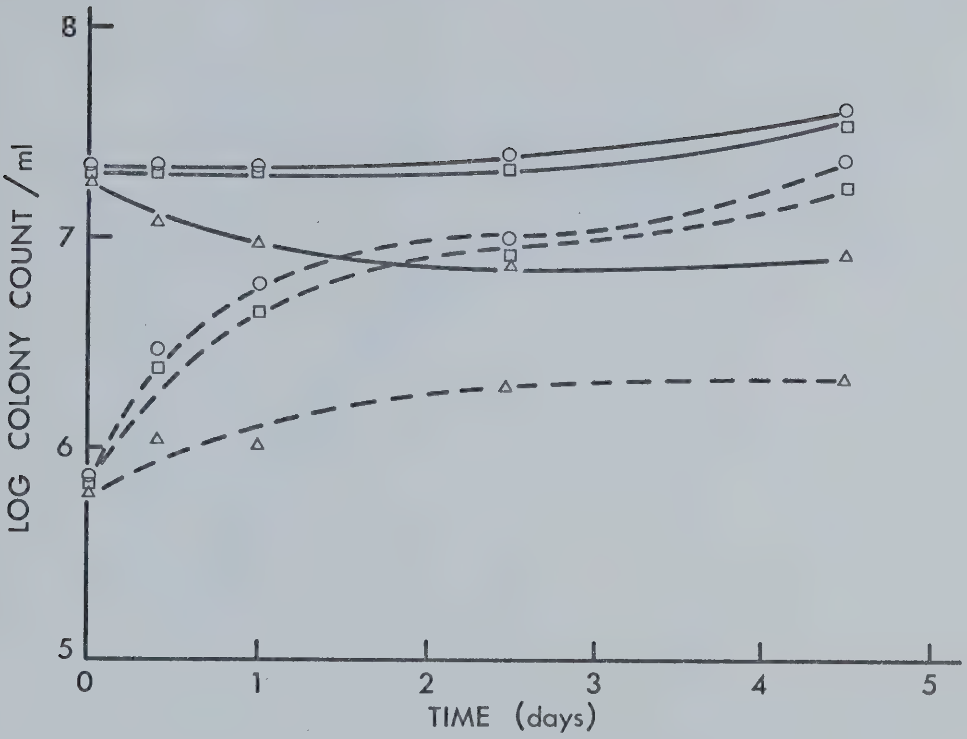

FIG. 53. Recovery of *Salmonella heidelberg* in tryptic soy broth of pH 7, 8 and 9 at 20°; organism grown in TSB at 37° and cold-injured for 4 days in TSB at 2°.

pH of broth: o, 7; □, 8; Δ, 9.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; ------, desoxycholate agar.

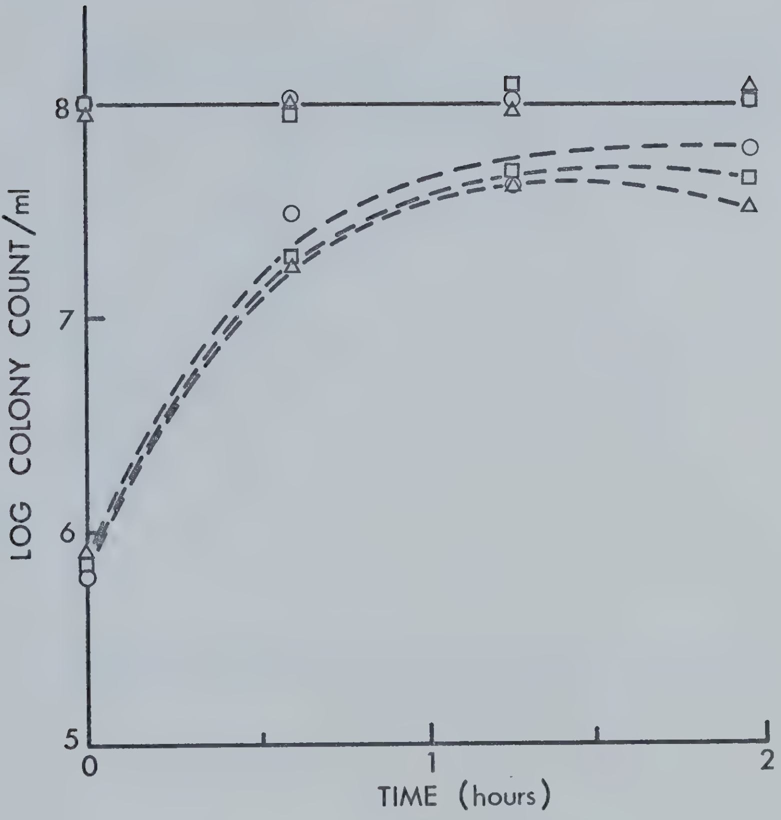

FIG. 54. Recovery of *Salmonella heidelberg* in tryptic soy broth plus chloramphenicol or nalidixic acid at 20°; organism grown in TSB at 37° and cold-injured for 5 days in TSB at 2°.

Recovery menstrua: o, TSB; Δ, TSB + chloramphenicol; □, TSB + nalidixic acid.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

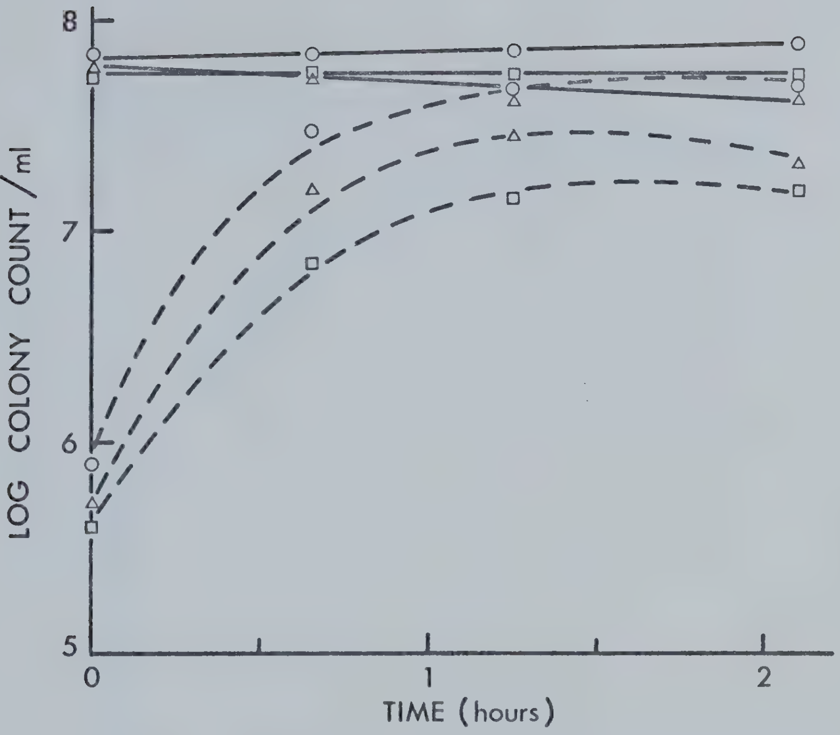

FIG. 55. Recovery of *Salmonella heidelberg* in tryptic soy broth plus rifampin or sodium azide at 20°; organism grown in TSB at 37° and cold-injured for 5 days in TSB at 2°.

Recovery menstrua: o, TSB; Δ, TSB + rifampin; □, TSB + sodium azide.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

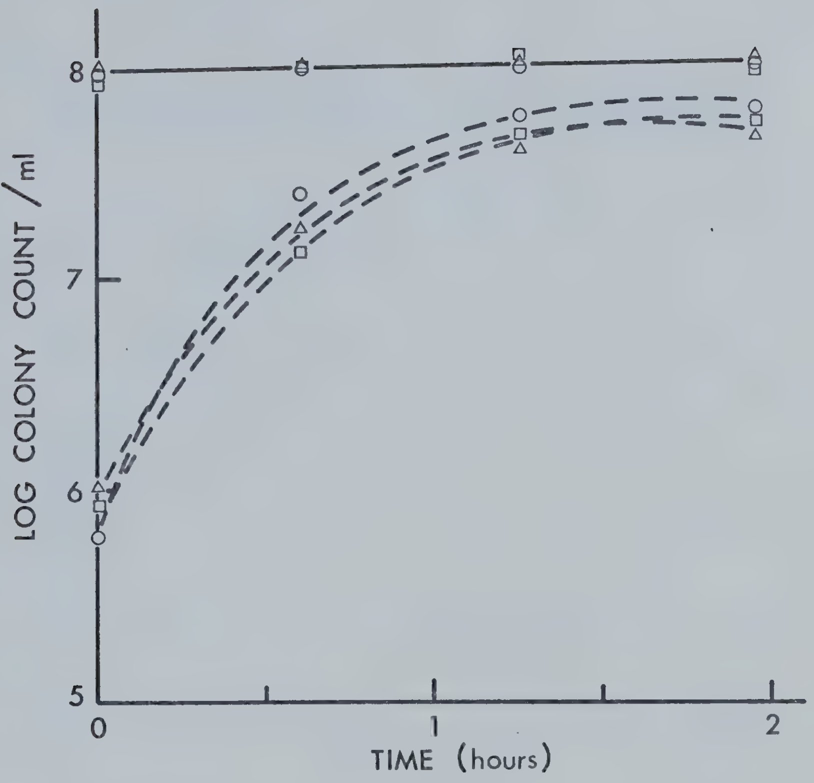

FIG. 56. Recovery of *Salmonella heidelberg* in tryptic soy broth plus cycloserine or gramicidin at 20°; organism grown in TSB at 37° and cold-injured for 5 days in TSB at 2°.

Recovery menstrua: o, TSB; Δ, TSB + cycloserine; □, TSB + gramicidin.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

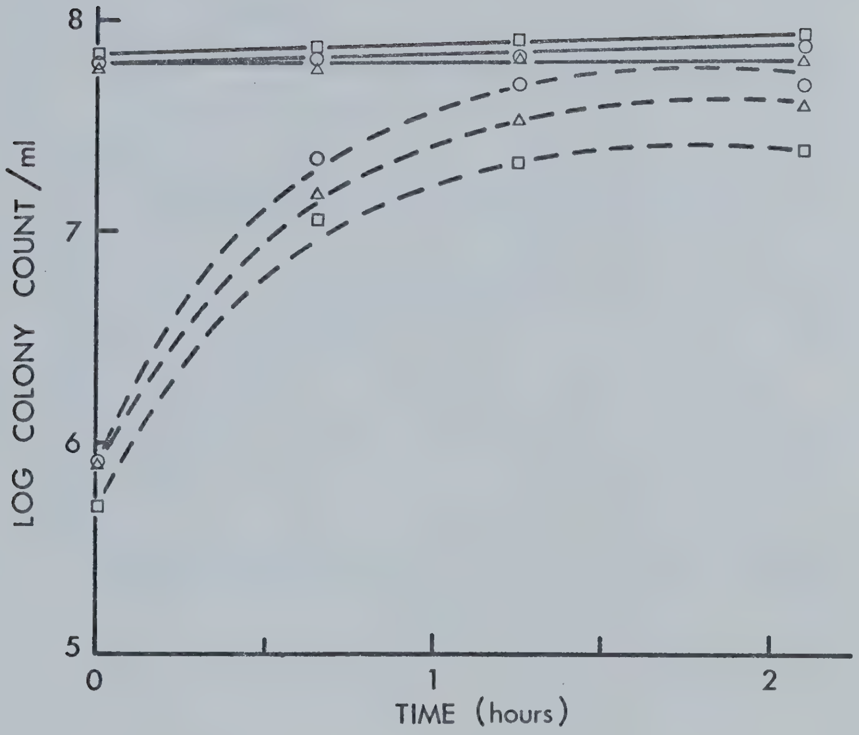

FIG. 57. Recovery of *Salmonella heidelberg* in tryptic soy broth plus penicillin or 2,4-dinitrophenol at 20°; organism grown in TSB at 37° and cold-injured for 5 days in TSB at 2°.

Recovery menstrua: o, TSB; Δ, TSB + penicillin; □, TSB + 2,4-dinitrophenol.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

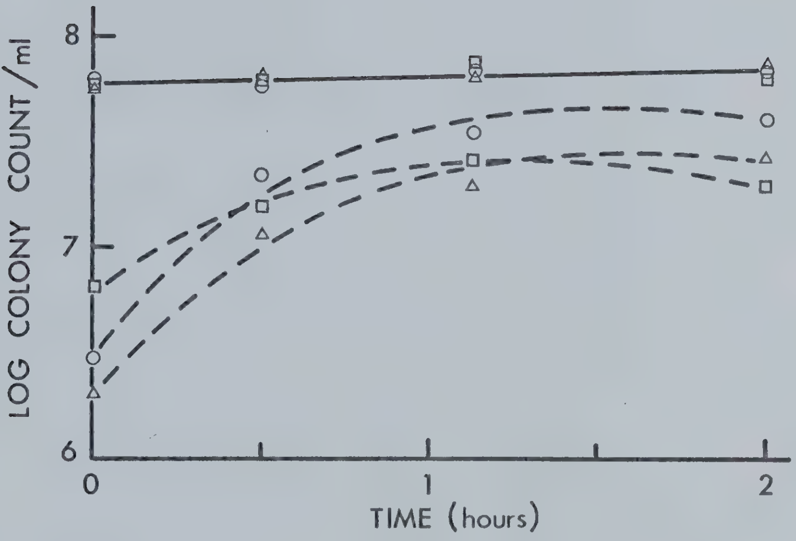

FIG. 58. Recovery of *Salmonella heidelberg* in glucose-salt broth, phosphate buffer or distilled water at 20°; organism grown in TSB at 37° and cold-injured for 4 days in TSB at 2°.

Recovery menstrua: o, glucose-salt broth; Δ, phosphate buffer; □, distilled water.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

fluorouracil. Fig. 59 shows that 2,4-dinitrophenol protects the cells both from injury and death compared with the control, TSB. Rifampin, on the other hand, increased both cell injury and death. Cell injury and death occurred much more drastically in the presence of penicillin (Fig. 60), while cultures containing hydroxyurea were very similar to the control. Oligomycin also gave similar results to the control, but sodium azide induced cell death (Fig. 61). The effect of chloramphenicol and fluorouracil was not significantly different from the control (Fig. 62). During cold storage, the inhibitors had been given ample of time to enter into the cells. All of the inhibitors with the exception of 2,4-dinitrophenol rendered the cells sensitive to desoxycholate agar. The recovery from such sensitivity was tested in the presence of residual inhibitors by transferring each of the cultures to a shaking water bath at 37° after 7 days of cold storage. Samples were taken from the recovery flasks at intervals and plated on TSY agar and desoxycholate agar. The results (Figs. 63 and 64) show that all cultures recovered from injury in the presence of the residual inhibitors tested.

C. TSB Grown Culture, Cold Stored in Distilled Water

The effect of cold storage in distilled water on *S. heidelberg* was also examined. The culture grown in TSB to exponential phase at 37° was harvested and washed twice with distilled water at 0-5° and finally stored in distilled water at 2°. After 4 days' storage, injured cells had developed and aliquots were then inoculated into tubes of TSB containing inhibitors pre-tempered at 0-5°. After the first sampling, the culture was then incubated in a water bath at 20° and subsequent recovery was followed. The result in Fig. 65 shows that chloramphenicol,

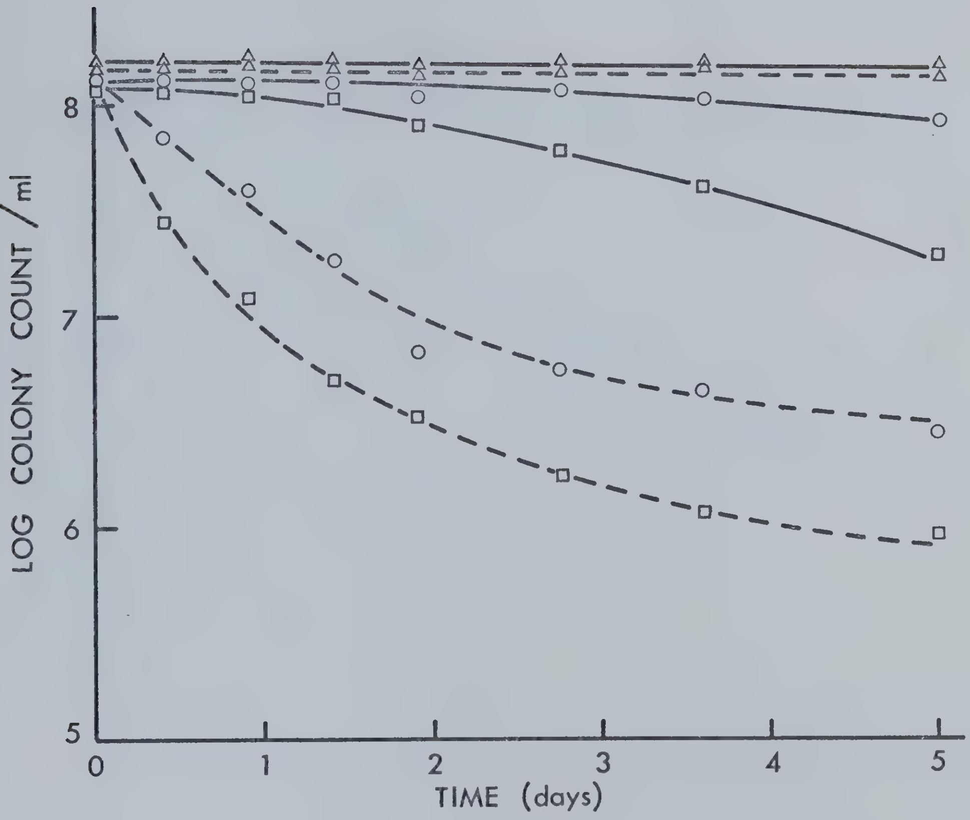

FIG. 59. Viability of *Salmonella heidelberg* in tryptic soy broth plus 2,4-dinitrophenol or rifampin at 2°; inoculum grown in TSB at 37°.

Storage menstrua: o, TSB; Δ, TSB + 2,4-dinitrophenol; □, TSB + rifampin.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

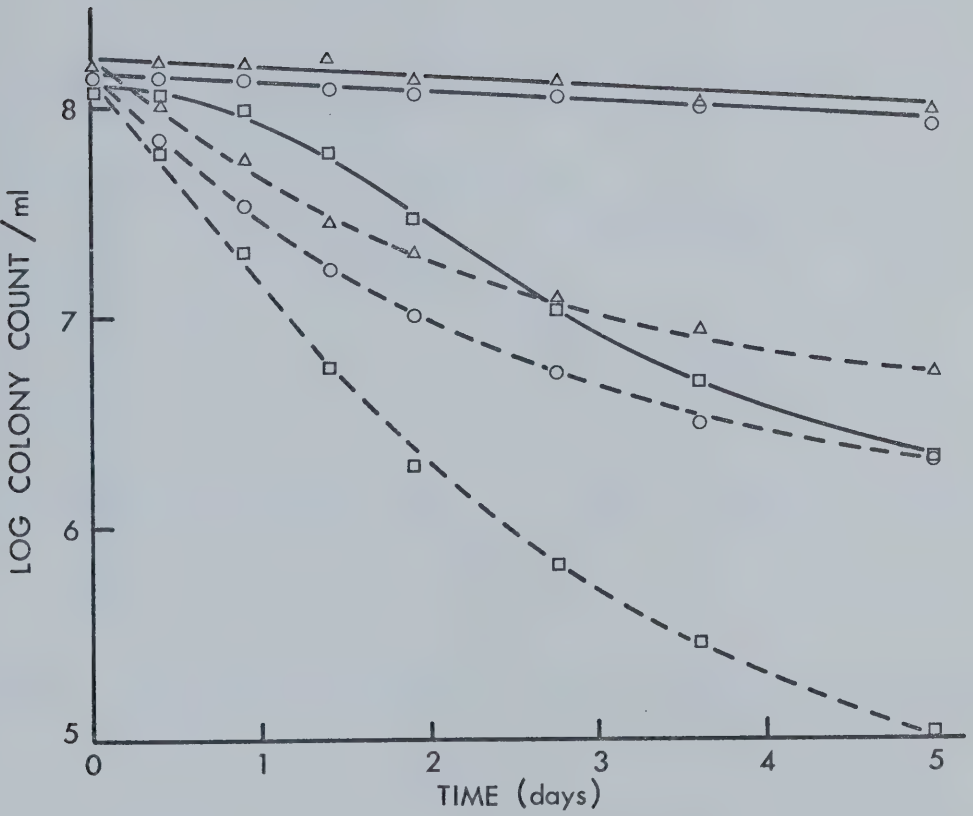

FIG. 60. Viability of *Salmonella heidelberg* in tryptic soy broth plus hydroxyurea or penicillin at 2°; inoculum grown in TSB at 37°.

Storage menstrua: o, TSB; Δ, TSB + hydroxyurea; □, TSB + penicillin.

Plating media: ——, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

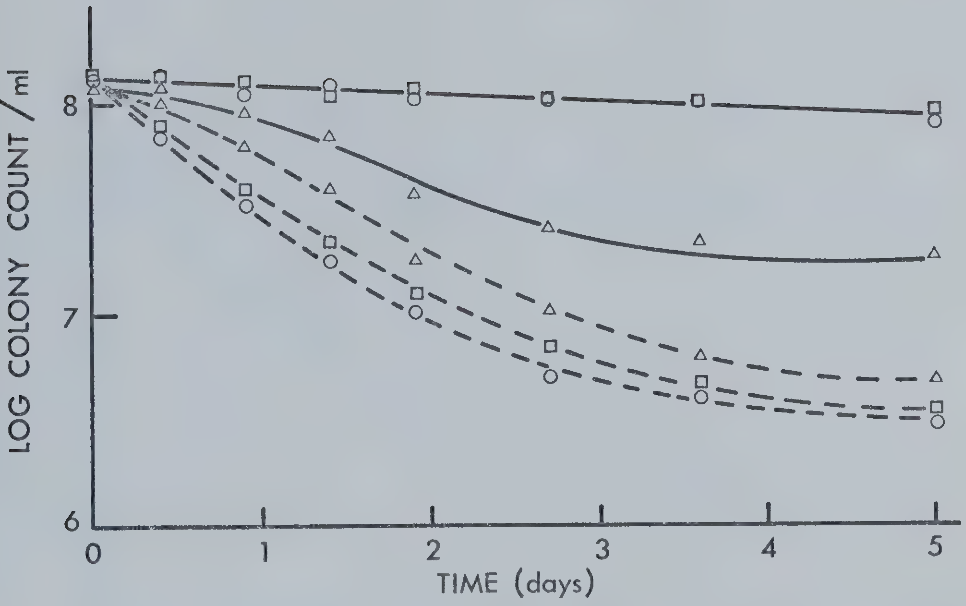

FIG. 61. Viability of *Salmonella heidelberg* in tryptic soy broth plus sodium azide or oligomycin at 2°; inoculum grown in TSB at 37°.

Storage menstrua: o, TSB; Δ, TSB + sodium azide; □, TSB + oligomycin.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

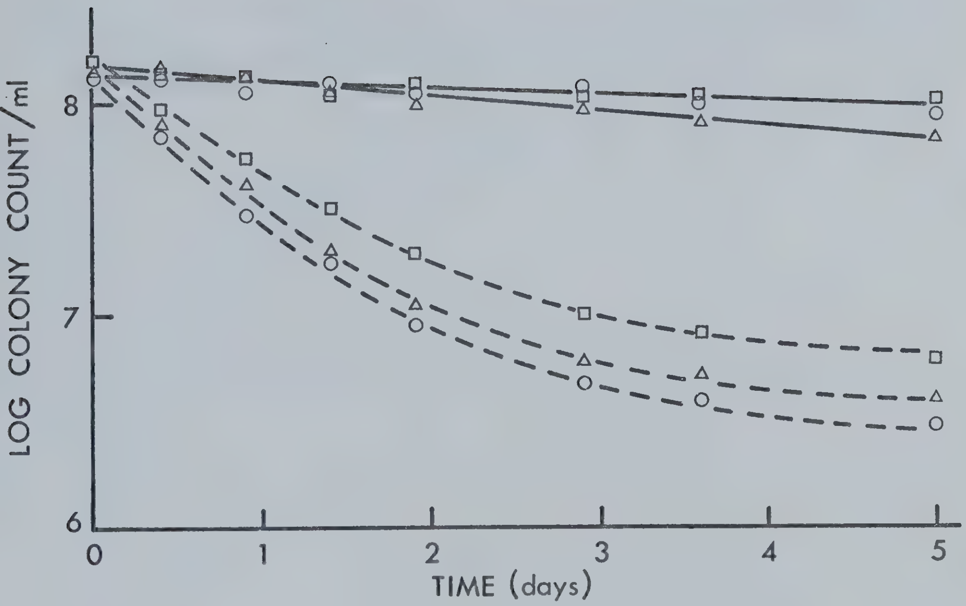

FIG. 62. Viability of *Salmonella heidelberg* in tryptic soy broth plus chloramphenicol or fluorouracil at 2°; inoculum grown in TSB at 37°.

Storage menstrua: o, TSB; Δ, TSB + chloramphenicol; □, TSB + fluorouracil.

Plating media: ———, tryptic soy agar + 0.5% yeast extract;
-----, desoxycholate agar.

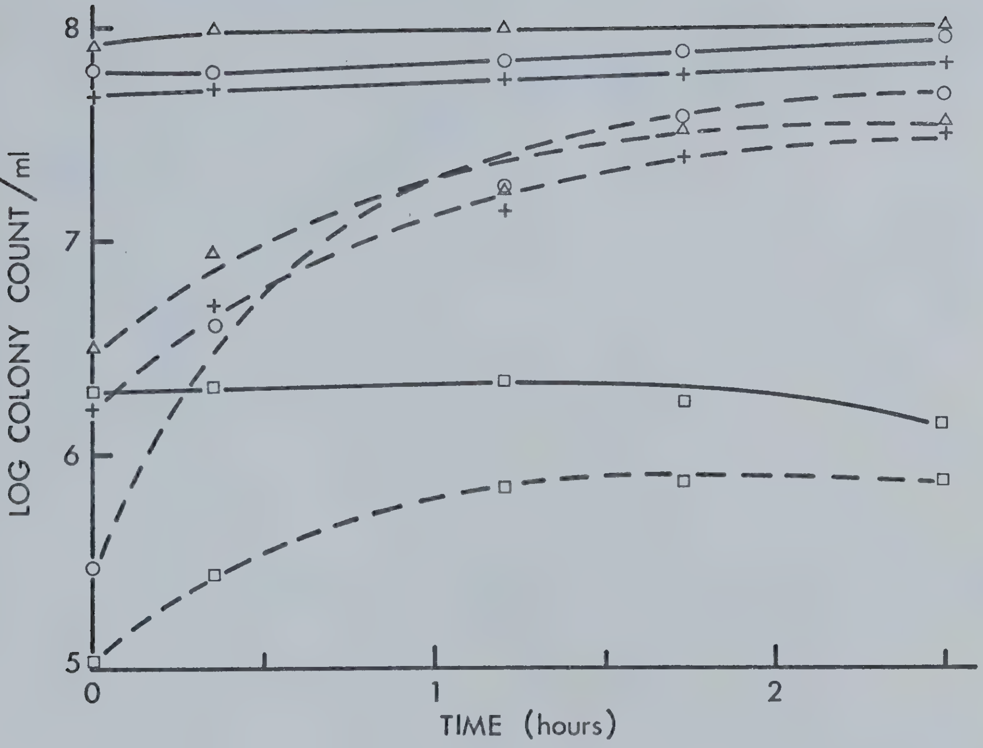

FIG. 63. Recovery of TSB grown *Salmonella heidelberg* at 37° after 8 days' storage in tryptic soy broth plus fluorouracil, chloramphenicol or penicillin at 2°.

Recovery menstrua: o, TSB; Δ, TSB + fluorouracil; +, TSB + chloramphenicol; □, TSB + penicillin.

Plating media: ——, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

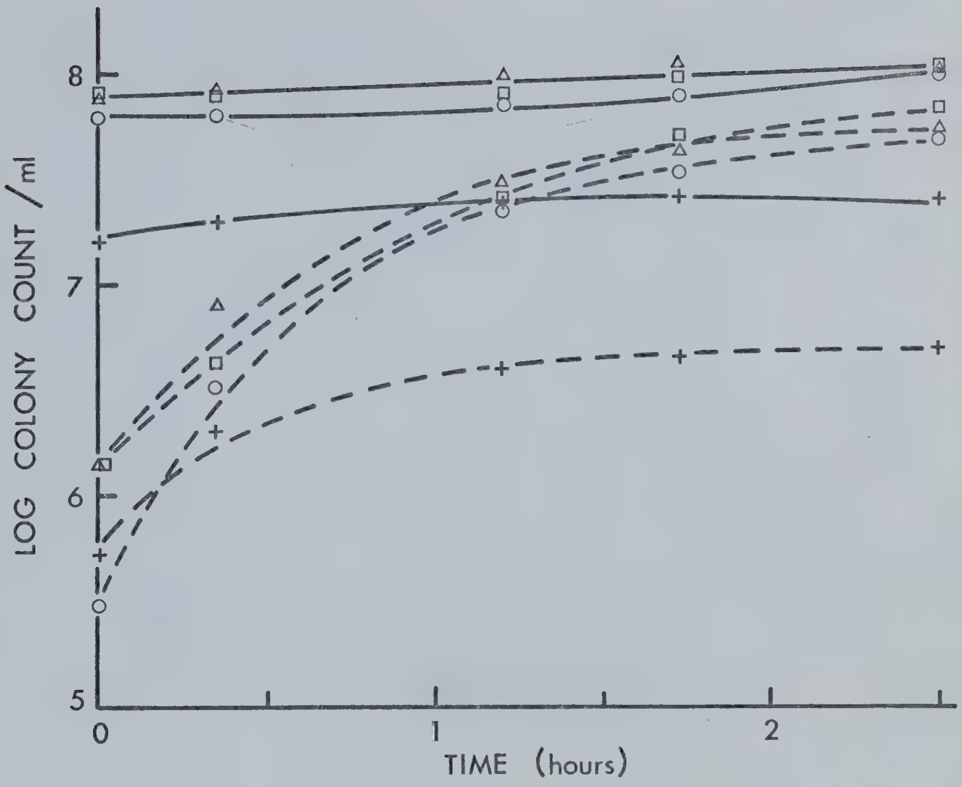

FIG. 64. Recovery of TSB grown *Salmonella heidelberg* at 37° after 8 days' storage in tryptic soy broth plus hydroxyurea, sodium azide or oligomycin at 2°.

Recovery menstrua: o, TSB; Δ, TSB + hydroxyurea; +, TSB + sodium azide; □, TSB + oligomycin.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

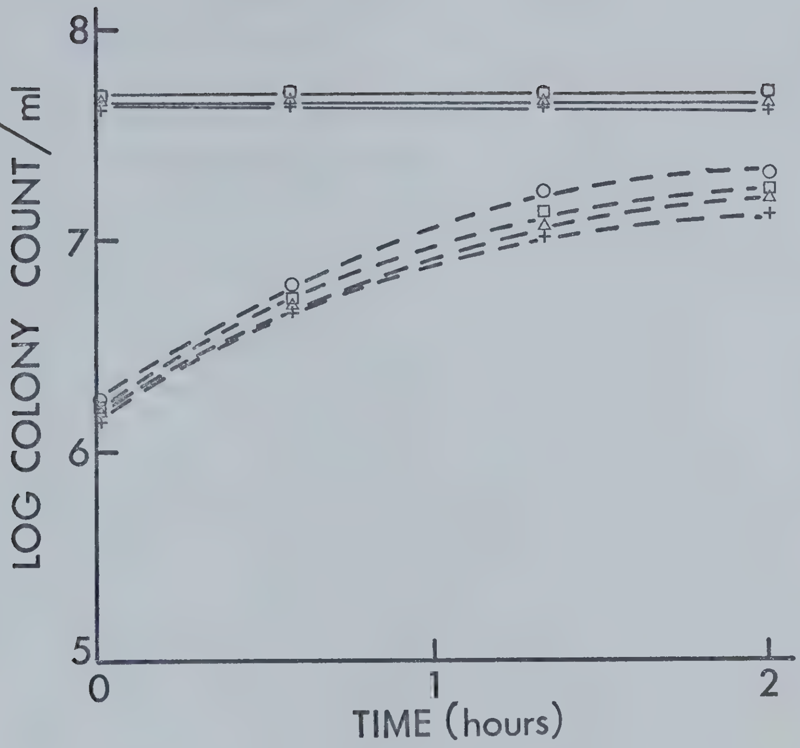

FIG. 65. Recovery of *Salmonella heidelberg* in tryptic soy broth plus nalidixic acid, fluorouracil or chloramphenicol at 20°; organism grown in TSB at 37° and cold-injured for 4 days in distilled water at 2°.

Recovery menstrua: o, TSB; Δ, TSB + nalidixic acid; □, TSB + fluorouracil; +, TSB + chloramphenicol.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

fluorouracil and nalidixic acid do not inhibit the recovery process in TSB. However, the recovery process was completely blocked by the presence of sodium azide and penicillin (Fig. 66). In addition, both inhibitors induced some cell death. Similar findings were observed when injured cells were allowed to recover in GSB instead of TSB. Recovery occurred in the presence of chloramphenicol, rifampin, fluorouracil and nalidixic acid (Fig. 67), but was inhibited completely by penicillin and drastically by 2,4-dinitrophenol (Fig. 68).

GSB has been shown to be very efficient in the recovery process. The ability of the individual components to aid in recovery was studied by suspending the injured cells in each of the ingredients in the same concentration and pH as in the GSB. The inoculated test solutions were incubated at 20° and samples were plated at time intervals to 2 hr (Figs. 69 and 70). All the components of GSB, phosphate, glucose, citrate, magnesium sulfate, ammonium sulfate and distilled water alone could not permit the recovery of injured cells. Instead, injured cells showed appreciable loss of viability in distilled water, glucose, ammonium sulfate and sodium citrate. Normal cells on the other hand, could survive very well in distilled water and practically no death could be detected on TSY agar up to 2 hr incubation at 20° (data not shown). The stress of low temperature imposed on the organism in distilled water appears to be more severe than in TSB. Under such a hypotonic cold environment, the cells apparently were rendered not only sensitive to desoxycholate agar but also osmotic stress. The recovery of injured cells to normal can probably be demonstrated with the return of normal osmotic resistance as well as desoxycholate tolerance. An experiment was conducted by inoculating injured cells

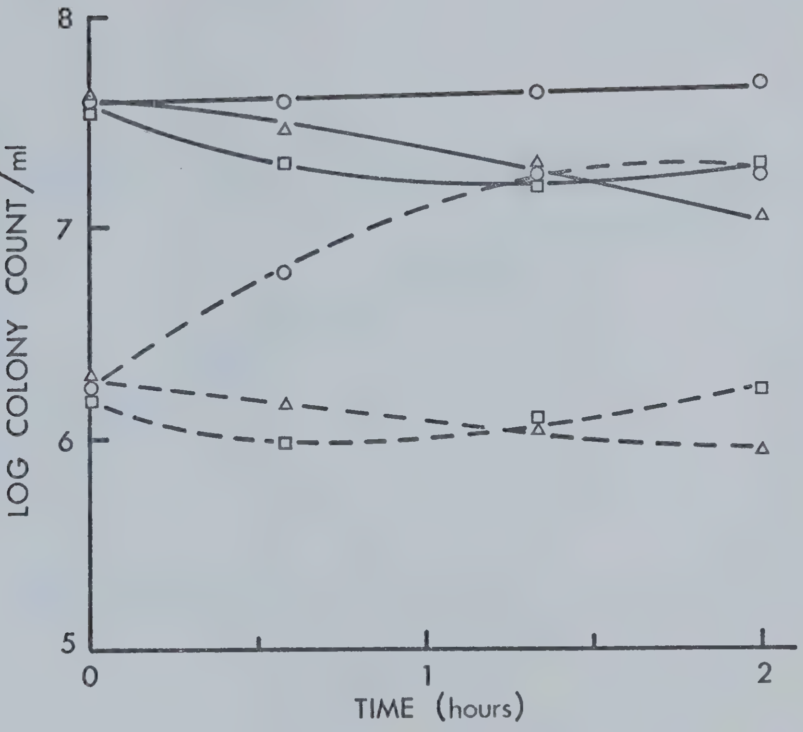

FIG. 66. Recovery of *Salmonella heidelberg* in tryptic soy broth plus penicillin or sodium azide at 20°; organism grown in TSB at 37° and cold-injured for 4 days in distilled water at 2°.

Recovery menstrua: o, TSB; Δ, TSB + penicillin; □, TSB + sodium azide.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

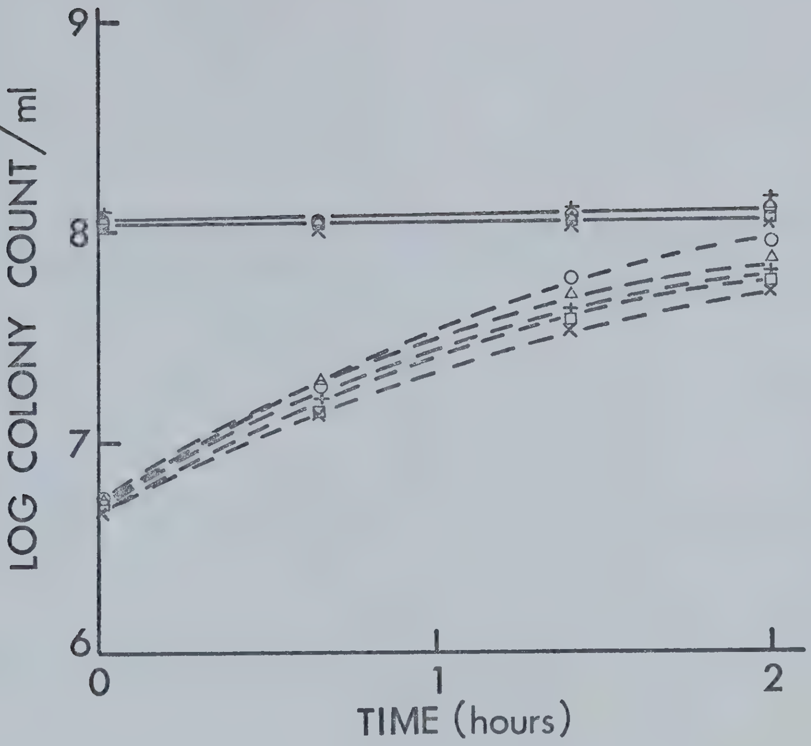

FIG. 67. Recovery of *Salmonella heidelberg* in glucose-salt broth plus rifamycin, fluorouracil, nalidixic acid or chloramphenicol at 20°; organism grown in tryptic soy broth at 37° and cold-injured for 4 days in distilled water at 2°.

Recovery menstrua: o, GSB; Δ, GSB + rifamycin; □, GSB + fluorouracil; +, GSB + nalidixic acid; x, GSB + chloramphenicol.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

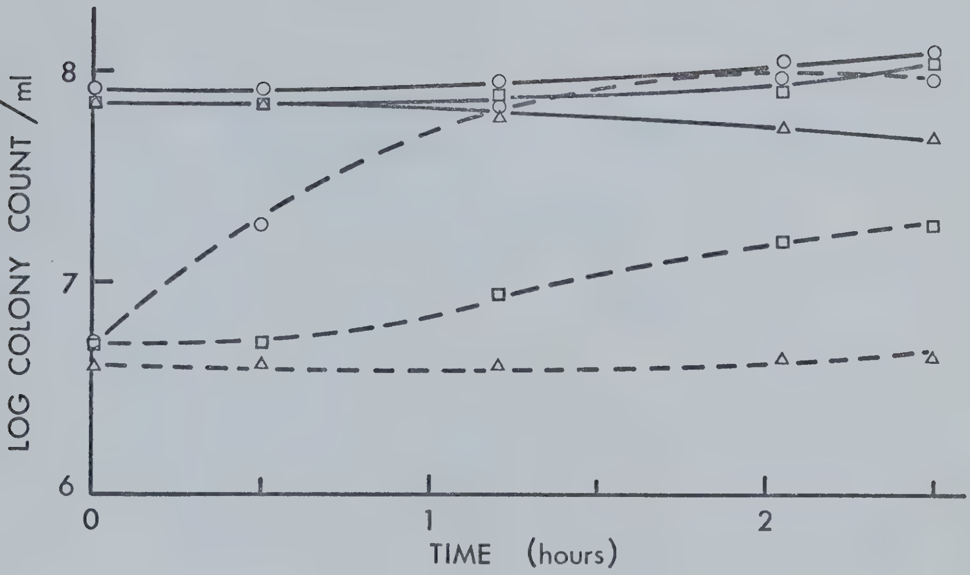

FIG. 68. Recovery of *Salmonella heidelberg* in glucose-salt broth plus penicillin or 2,4-dinitrophenol at 20°; organism grown in tryptic soy broth at 37° and cold-injured for 4 days in distilled water at 2°.

Recovery menstrua: o, GSB; Δ, GSB + penicillin; □, GSB + 2,4-dinitrophenol.

Plating media: ———, tryptic soy agar + 0.5% yeast extract;
-----, desoxycholate agar.

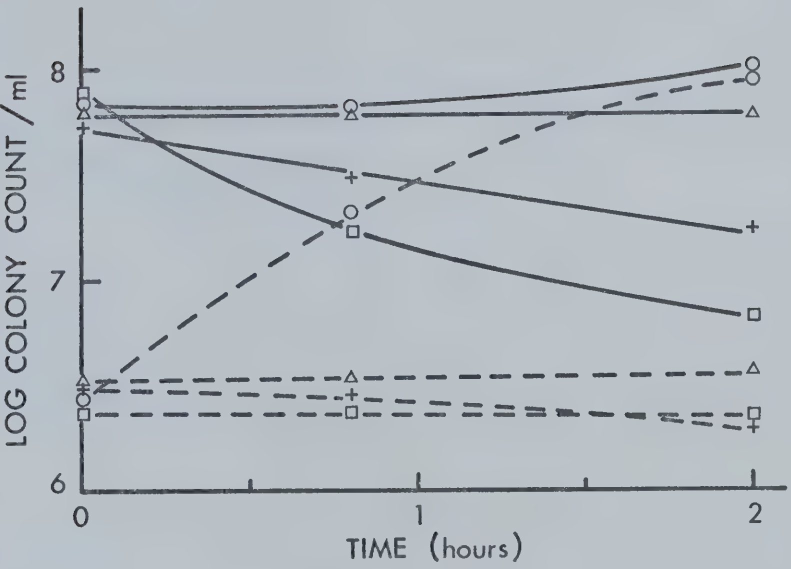

FIG. 69. Recovery of *Salmonella heidelberg* in different components of glucose-salt broth at 20°; organism grown in tryptic soy broth at 37° and cold-injured for 4 days in distilled water at 2°.

Recovery menstrua: o, GSB; Δ, phosphate buffer; +, glucose;
□, distilled water.

Plating media: ———, tryptic soy agar + 0.5% yeast extract;
-----, desoxycholate agar.

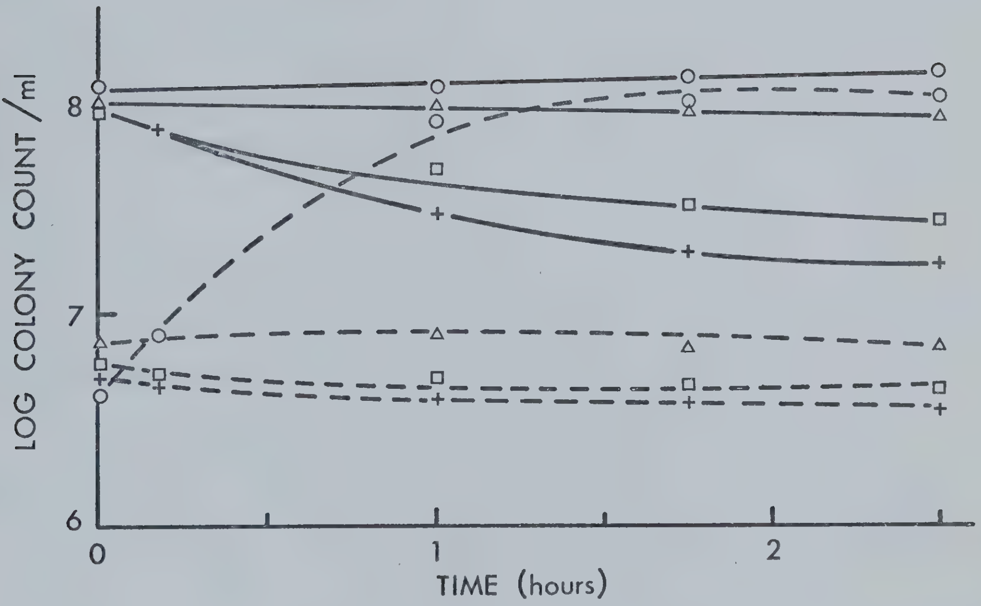

FIG. 70. Recovery of *Salmonella heidelberg* in different components of glucose-salt broth at 20°; organism grown in tryptic soy broth at 37° and cold-injured for 4 days in distilled water at 2°.

Recovery menstrua: o, GSB; Δ, magnesium sulfate; □, ammonium sulfate; +, sodium citrate.

Plating media: ———, tryptic soy agar + 0.5% yeast extract; -----, desoxycholate agar.

into GSB which was then incubated at 25° for recovery. At intervals samples were plated on TSY agar and desoxycholate agar so as to determine the percentage of cells that had retained the tolerance to desoxycholate and at the same time, the tolerance to osmotic stress. Osmotic tolerance was determined by harvesting the culture sample which was then washed twice with cold distilled water. The suspension was finally incubated at 25° for 1 hr. The initial and final counts on TSY agar was determined before and after the incubation. The results (Fig. 71) show that recovery was accompanied by both a return of resistance to desoxycholate agar and osmotic stress, both of which followed each other until 70 min of incubation, after which the former rate decreased whereas the latter proceeded at an undiminished rate.

Previous experiments on GSB grown *S. heidelberg* showed that injured cells had increased permeability to ANS. The ability of RNase to penetrate TSB grown culture after cold storage was studied by determining their survival in phosphate buffer (0.063M, pH 7.2) during incubation with the enzyme at 25° (Fig. 72). Normal cells grown in TSB at 37° were harvested, washed with distilled water and resuspended in phosphate buffer plus ribonuclease (100 μg/ml). The cell suspension was then incubated at 25° and viability was determined by plating samples on TSY agar at intervals. The normal culture was able to survive well up to 2 hr of incubation. When the organism was injured in distilled water for 4 days at 2°, it showed rapid death within 30 min. When the injured culture was allowed to recover for 1.5 hr in GSB at 25°, it retained its ability to survive under these conditions.

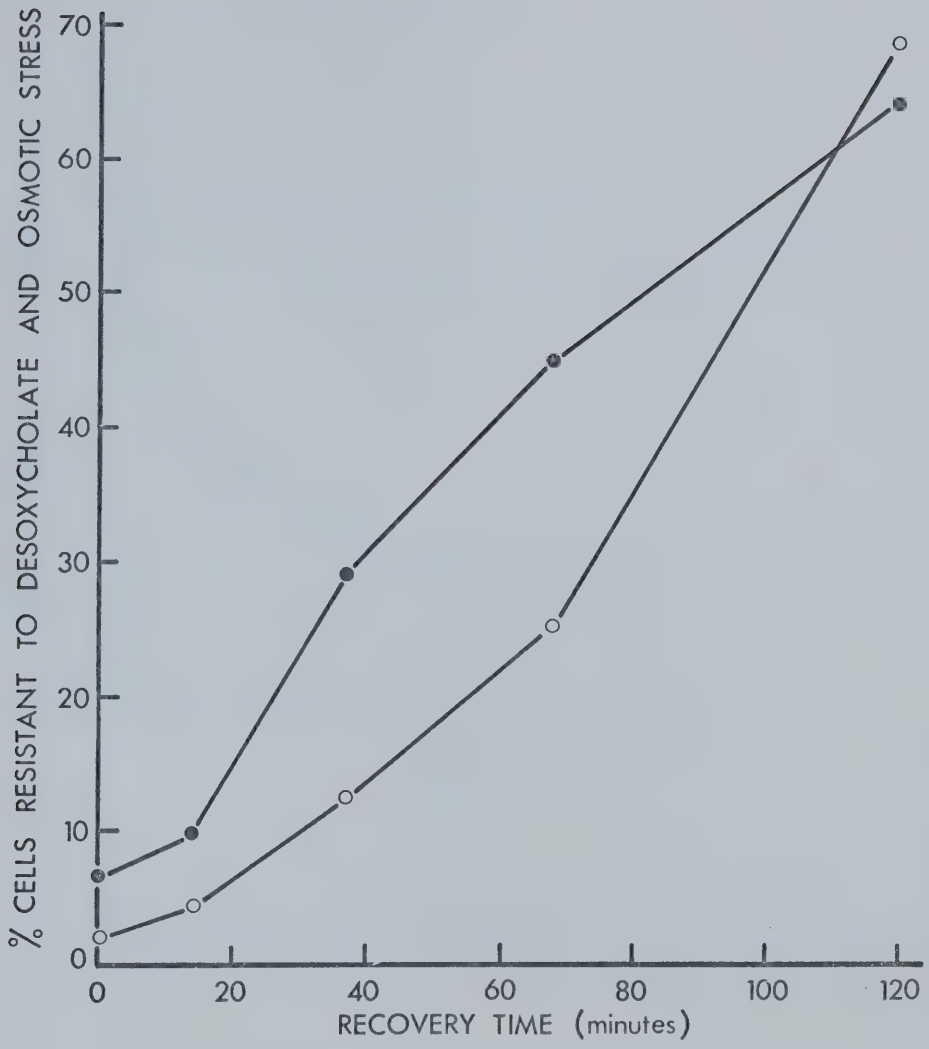

FIG. 71. Recovery of osmotic tolerance and resistance to desoxycholate agar in *Salmonella heidelberg* incubated at 20° in glucose-salt broth. The culture grown in tryptic soy broth at 37° was harvested, washed and stored in distilled water at 2° for 5 days after which they were allowed to recover in GSB at 20°. At intervals samples were taken from the recovering culture and tested for their tolerance to desoxycholate agar and osmotic stress.

Symbols: ●, % cells resistant to desoxycholate agar; ○, % cells resistant to osmotic stress determined by the % survivals of washed cell samples after incubation in distilled water at 25° for 1 hr when plated on tryptic soy agar + 0.5% yeast extract.

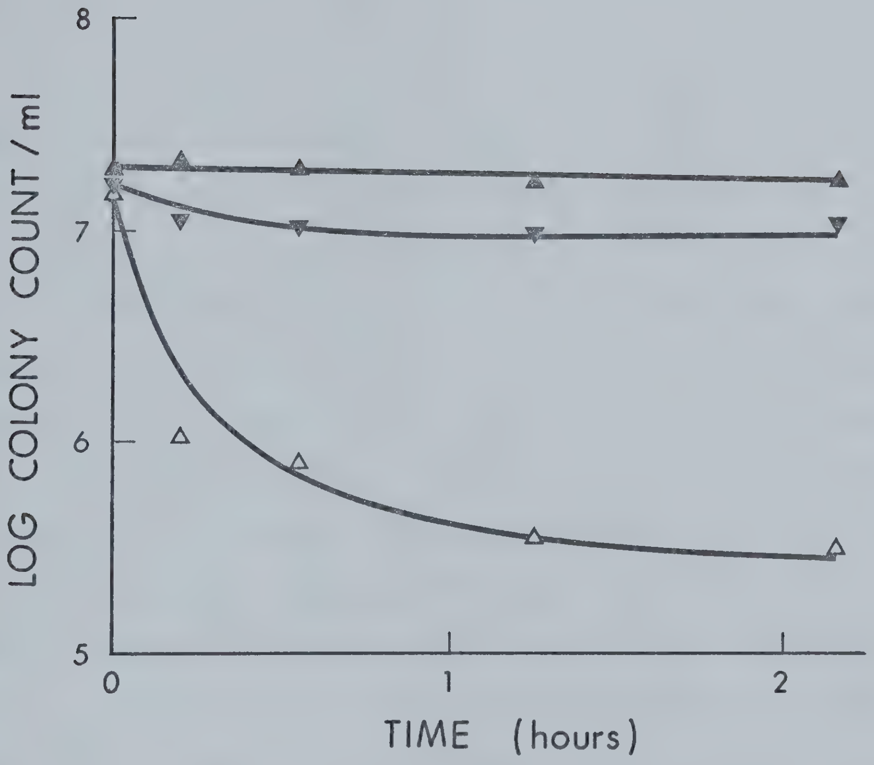

FIG. 72. Survival of *Salmonella heidelberg* in phosphate buffer plus ribonuclease at 25°; organism grown in tryptic soy broth at 37° (▲), cold-injured for 4 days in distilled water at 2° (△), and allowed to recover for 1.5 hr in glucose-salt broth at 25° (▼).

Plating medium is tryptic soy agar + 0.5% yeast extract.

DISCUSSION AND CONCLUSIONS

The initial aim of the present study was to determine the effect of sub-optimal temperatures on the growth and viability of salmonellae in tryptic soy broth (TSB) and glucose-salt broth (GSB) at pH 5, 6 and 7, using a temperature gradient incubator.

The data indicated that TSB at pH 6 and 7 could support abundant growth at 6° but not at 4°, while that at pH 5 could support slight growth at 14° and abundant growth at 16°. This finding reinforced most workers' conclusions that food should be held below 5° at all times to prevent the growth of salmonellae (Prescott and Geer, 1936; Prescott and Tanner, 1938; Angelotti *et al.*, 1961; Matches and Liston, 1972). It should be emphasized at this point that although growth was observed at 6° in TSB at pH 6 and 7, this occurred under ideal conditions for salmonellae. A complete nutritional medium was used, free from competing microflora and natural inhibitors that would be present in the actual food product. It is not possible to extrapolate the behavior that occurs in a laboratory medium to that in a food product or the environmental situation that actually exists in a food processing establishment. However, the minimal growth temperatures were derived under ideal laboratory conditions and, as such, would represent the absolute minima below which no growth of salmonellae will occur.

Matches and Liston (1968) reported that as the temperature was decreased towards the minimum, the most obvious effect on the growth of salmonellae was an extension of the lag phase. This lag could be as

long as 5 days in case of *S. heidelberg* and 26 days in case of *S. typhimurium* and *S. derby*. Our results did not show such a long lag. Instead, growth in TSB at pH 6 and 7 was rapid even at 6° and reached the maximum on the tenth day of incubation (Figs. 6, 7, 15, 16, 18 and 19). The long lag of low temperature found by Matches and Liston could be due to the fact that they used a smaller inoculum and an inoculum obtained from the stationary phase. In agreement with our results (Figs. 5, 14 and 17), they found that salmonellae were unable to grow over a temperature range of 2-12° at pH 5 (Matches and Liston, 1972).

An interesting finding is that the survival of salmonellae grown at low temperatures is much greater than organisms grown at higher temperatures. This is probably related to the change in pH of the medium which is more noticeable at higher temperatures, augmented by higher temperatures of incubation (Tables 1, 2 and 3). Thus cells grown in TSB at pH 5 and 7 having their final pH on the critical acid or alkaline side respectively at or above 21° died off rapidly. The death rate was enhanced at temperatures further above 21°. On the other hand, cells grown at pH 6 did not yield enough acids to be lethal and their survival was great even at higher temperatures. The survival of salmonellae at low temperatures perhaps also depends on the previous history or make-up of the organism. Smith *et al.* (1974) who also found that higher temperatures enhanced death of gram-negative bacteria at their stationary phase, proposed that a prerequisite for long-term survival of vegetative cell cultures was completion of secondary metabolism. Thus environmental factors such as temperature, pH, redox potential, trace metal and inorganic phosphate concentrations that permit good yields of secondary substances should favor longevity;

those that suppress secondary metabolism should cause early death (Demain, 1968; Weinberg, 1970, 1971a,b; Smith *et al.*, 1974).

S. heidelberg grew much more slowly and gave less cell yield in GSB than in TSB at all temperatures and pH levels. The organism could grow in GSB at low temperatures of 11, 9 and 9° at pH 5, 6 and 7 respectively (Figs. 20-25). But incubation was carried out in a static temperature gradient incubator and the oxygen availability might be limited and become critical in the initiation of growth in the minimal medium at low temperatures. In fact, when aerated the organism could grow well at 7° and slightly at 6° in GSB at pH 7 (Figs. 29 and 33). On the other hand the reason for the lower minimum temperature of growth in GSB compared with TSB at pH 5 is not clear. Growth in GSB at pH 5 and 6 yielded comparatively more acids than in TSB (Table 4) leading to a faster death rate in the stationary phase at temperatures above 21°. However, growth in GSB at pH 7 yielded a final pH around neutrality and, hence unlike that in TSB at pH 7, led to long survival of the organism even at higher temperatures.

The data on the effect of temperature on generation time and growth rate of *S. heidelberg* in GSB indicates that the organism shows the temperature response of a typical mesophile (Ingraham, 1958). At temperatures below 18°, growth rates are greatly slowed and deviated from the Arrhenius equation (Fig. 31). At these low temperatures cell yield is no more constant and is independent of growth rate. Instead it falls off precipitously. Forrest (1967) and Ng (1969) found similar results with *Strep. faecalis* and *E. coli* respectively and concluded that decrease in cell yield with decrease in temperature was a result of uncoupling of energy utilization from energy production. This was due to the limitation

of certain biosynthetic processes and not due to the limitation of energy production nor decreased permeability. The present data show that ATP synthesis was not decreased at low temperatures nor was the leakage of ATP from cells stored at 7° significant (Figs. 28 and 29). Therefore, the failure of sugar transport coupled with loss of permeability control at low temperatures as a possible basis for the minimal growth temperature of bacteria (Farrell and Rose, 1967a) is not supported.

The above hypothesis, that biosynthesis limits growth at low temperatures, is further supported by the findings of Das and Goldstein (1968) and Friedman *et al.* (1969). The former worker demonstrated that at 0° protein synthesis in *E. coli* slows progressively and eventually stops, while RNA synthesis continues at an undiminished rate. The cause of low temperature defect on protein synthesis appears to be an inability of free ribosomes to attach to mRNA. The evidence suggests that a ribosome has to be activated once it has been released from the 3' end of a mRNA strand, in order to permit its functional reattachment at the 5'-end of a new operon. This activation apparently proceeds too slowly at 0° to be effective in completion with inactivation processes. The low temperature effect in protein synthesis could explain the well known phenomenon of minimal growth temperature. Friedman *et al.* (1969) found that lowering the temperature of *E. coli* to 8° resulted in an accumulation of ribosomal subunits due to a block in the initiation of protein synthesis while the elongation of proteins initiated before the temperature shift continues until they are completed. This low temperature ribosomal subunit accumulation has a sharp cut-off between 8° and 10° and thus *E. coli* can initiate protein synthesis at 10° and above but not at 8° or below this temperature. However, most activities including RNA and

DNA synthesis continue at 7°. They suggested that the failure to initiate the synthesis of new proteins is responsible for failure to carry out cell division; a process which naturally requires new proteins.

As mentioned earlier, growth rate of *S. heidelberg* is greatly diminished towards the minimal growth temperature and deviates from the linearity of the Arrhenius plot. Most workers point out that biosynthesis and not catabolism is the limiting factor at low temperatures. It would be of interest to find out the effect of temperature on the rate of protein, RNA and DNA synthesis during balanced growth of *S. heidelberg*. An Arrhenius plot of the data might show which biosynthetic process is affected at low temperatures.

In any study on the effect of low temperature on micro-organisms, the phenomenon of cold shock must be recognized and taken into account. It is a well documented phenomenon that may result in damage or death to micro-organisms upon sudden chilling of a dilute suspension of bacteria (Farrell and Rose, 1967b). Shock occurs only under specialized conditions but when these conditions are met survival can drop as much as 10,000-fold. Susceptibility to cold shock is usually found only in bacteria from exponential phase cultures (Gorill and McNeil, 1960) and is dependent on rapid chilling. The occurrence of cold shock is also affected by the concentration of cells, the medium in which the cells are grown and in which they are suspended during chilling (Gorill and McNeil, 1960; Houghtby and Liston, 1965; Strange, 1964). In the present work cold shock did occur when exponentially growing *S. heidelberg* in GSB was inoculated into fresh GSB pretempered at 5°, no matter whether the inoculum was obtained directly from the growth vessel at 37° or by harvesting the culture at

18° and resuspending in a small volume of GSB at room temperature. As many as 2.5 log cycles of cells were damaged and rendered incapable of growth on TSYA. However, when the chilled cells were enumerated on GSA instead, cold shock was not demonstrated. The ability of the chilled cells to grow on minimal medium rather than on the complex rich medium is the reverse of the phenomenon of 'metabolic injury' reported on micro-organisms subjected to freezing (Straka and Stokes, 1959; Postgate and Hunter, 1963; Moss and Speck, 1966a; Kuo and Macleod, 1969) or subjected to sublethal heating (Nelson, 1943; Allwood and Russell, 1966; Russell and Harries, 1968). However, it is similar to the 'minimal medium recovery' effect observed in radiation sensitive organisms (Ganesan and Smith, 1970) and sublethally heated *Salmonella* (Gomez *et al.*, 1973).

Upon further incubation at 5°, the chilled *S. heidelberg* gradually recovered from its sensitivity to TSYA (Fig. 46). Sato and Takahashi (1969) reported that cold shocked *E. coli* could recover in magnesium solutions at a temperature as low as 3° and the recovery process required energy synthesis. However, in this study, recovery of *S. heidelberg* from cold shock occurred in the presence of 2,4-dinitrophenol (Fig. 48). The difference in these findings is probably due to the fact that the recovery menstruum employed in this experiment was glucose-salt broth rather than magnesium solution. Although 2,4-dinitrophenol is a potent inhibitor of oxidative phosphorylation, it does not inhibit the production of ATP via substrate level phosphorylation, for example, from glucose in *Staph. aureus* (Iandolo and Ordal, 1966) and *S. typhimurium* (Pierson, 1970). The results also showed that rifamycin and rifampin which are potent inhibitors of RNA synthesis,

irreversibly binding to DNA-dependent RNA polymerase, inhibited the recovery of chilled *S. heidelberg* (Fig. 49). Thus RNA synthesis is required in the recovery process at 5°. As mentioned earlier, RNA synthesis can proceed at temperatures as low as 0 and 7° in *E. coli* (Das and Goldstein, 1968; Friedman *et al.*, 1969).

Cold shock has been shown to alter membrane permeability leading to leakage of U.V.-absorbing compounds from *A. aerogenes* (Strange and Dark, 1962) and stimulate auto-degradation of RNA in the organism (Strange and Postgate, 1964). The recovery process of *S. heidelberg* is much faster at 20° than at 5° (Fig. 46). Similarly that of *E. coli* is favored at 30° than at 3° and is facilitated by magnesium ions (Sato and Takahashi, 1969). Magnesium ions are well recognized as cofactors of many enzymatic activities, an important stabilizer of ribosomes (McQuillen, 1962) and are able to diminish RNA degradation in heat stressed *A. aerogenes* (Strange and Shon, 1964). They also affect membrane porosity (Scherrer and Gerhardt, 1973).

Unlike cells grown in GSB, *S. heidelberg* grown in TSB was more resistant to cold shock and no significant difference between counts on GSA and TSYA was found (data not shown). This is in agreement with the finding of Strange and Ness (1963) that susceptibility to cold shock in some bacteria decreases when the organisms are grown in nutritionally complex media rather than in chemically defined media. Growth in nutritionally complex media in general leads to an increased synthesis of unsaturated fatty acids by bacteria (Kates, 1964). Rose (1968) suggested that susceptibility to cold shock in Gram-negative bacteria may be a function of the content of unsaturated fatty acid residues in the membrane lipids.

It is concluded that cold shock of *S. heidelberg* in GSB should be interpreted with caution as the chilled organism showed cellular injury rather than death. Injury is probably due to altered membrane permeability and ribosome integrity and is manifested as loss of ability to form colonies on TSYA. The injury is reversible and can be repaired when RNA synthesis takes place. The results question the use of rich media as pre-enrichment procedures for the isolation of chilled foodborne pathogens and it is suggested that GSB is probably more appropriate for the pre-enrichment step in the isolation of chilled salmonellae.

During prolonged storage of GSB grown or TSB grown *S. heidelberg* at 0-5° in GSB or TSB respectively, the organism was injured and rendered unable to grow on desoxycholate agar or MacConkey agar (Fig. 32). This observation is different from cold shock, as both GSB grown and TSB grown cultures showed the same injury; viable counts on TSYA and GSA were practically the same and both media supported many more colonies than desoxycholate agar.

The phenomenon of increased nutritional requirement was not observed with GSB grown *S. heidelberg* when stored at 5°. Counts on TSYA and GSA were similar during recovery as was the rate of recovery in TSB and GSB (Figs. 34 and 35). These findings differ from results with *E. coli* and *S. anatum* subjected to freezing, both of which showed increased nutritional requirements or better recovery in a rich medium (Moss and Speck, 1966a,b; Ray *et al.*, 1972).

Analysis of GSB grown *S. heidelberg* did not show any appreciable degradation of RNA, DNA and protein during storage at 2°. The recovery process of GSB grown and TSB grown cultures from injury was

similar. Both can recover in the presence of inhibitors of RNA, DNA, protein, mucopeptide or ATP synthesis. The endogenous reserve of such cultures probably plays an important role in the recovery process, as both can recover very well in phosphate buffer. On the other hand, ATP synthesis during recovery from injury caused by freezing or freeze-drying in *S. anatum* has been reported (Ray *et al.*, 1971 and 1972). The difference in results could be due to the fact that freezing and freeze-drying are more severe stresses causing metabolic injury of the cells with increased nutritional requirements or could be due to the use of different plating media.

As mentioned earlier, some microbial activities, including RNA and DNA synthesis, can continue even below the minimal growth temperature (Das and Goldstein, 1968; Friedman *et al.*, 1969). Such unbalanced metabolism could also occur in *S. heidelberg* at 0-5°, but could not be the cause of injury observed in the present study. Firstly, injury occurred not only at sub-minimal growth temperatures but also at 6°, about 0.7° above the minimal growth temperature of *S. heidelberg*. At this temperature some growth and presumably balanced metabolism occurred in TSB. Secondly, addition of antimicrobial inhibitors to the storage menstrua at 2° did not increase or decrease the percentage of injury significantly, with the exception of 2,4-dinitrophenol (Figs. 59-62). Friedman *et al.* (1969) found that incubation of *E. coli* at 0-5° led to accumulation of ribosomal subunits. Such accumulation was complete within 3 hrs at 5°, but this probably can not be related to the injury of *S. heidelberg* which developed gradually and became significant only after several days of incubation at the same temperature. Moreover, no significant difference in the amount of

injury was noted between cultures chilled in TSB and in TSB plus chloramphenicol which, in its presence, will halt the accumulation of ribosomal subunits at low temperatures.

The presence of penicillin during cold storage of *S. heidelberg* in TSB induced rapid death of the organism. This is probably due to the fact that under this condition, cell wall mucopeptide was not synthesized. The mechanism of the protective effect of 2,4-dinitrophenol on chilled *S. heidelberg* is not clear, but is probably due to a physical rather than a biochemical effect, since oligomycin, also an inhibitor of ATP synthesis, does not protect the cells from injury (Figs. 59 and 61).

Although the exact mechanism of cold injury of TSB grown or GSB grown *S. heidelberg* when stored in TSB or GSB respectively is not clear, it appears that cold injury produces some kind of damage to the cell envelope of the organism resulting in increased permeability. There is leakage of U.V.-absorbing compounds from the organism during cold storage and the dye, ANS, which normally cannot enter the cells, can now do so (Figs. 44 and 45). The recovered cells regained their impermeability to the dye. The fact that injured cells are rendered sensitive to desoxycholate and MacConkey agar would indicate that the outer structure of the cells was damaged. The agars contain respectively desoxycholate and bile salt which are surface-active compounds. It is known that in the gram-negative bacteria, the outer lipopolysaccharide layer of the cell wall protects the mucopeptide layer of the wall and the lipo-protein layer of the membrane against the action of hydrolytic enzymes and surface-active agents respectively. Hence normal cells are not sensitive to desoxycholate or MacConkey agar. Cold storage probably induces some steric or chemical change of the

lipo-polysaccharide layer and allows the surface-active agents to come in contact with the lipo-protein layer of the membrane. The damage to the cell envelope is reversible and can be recovered. The fact that recovery is favored at pH 6-8 and is much faster at 35° suggests the process may involve an enzymatic reaction.

The stress imposed on the TSB grown *S. heidelberg* during cold storage in distilled water appears to be more severe than in TSB. Under such a hypotonic environment, the cells are constantly subjected to both cold temperature and osmotic stress. The organism becomes sensitive to desoxycholate agar and has an increased permeability, as evidenced by the ability of RNase to enter the cells. Permeability to RNase is lost when the cells are allowed to recover. However, unlike cells stored in TSB, the injured cells cannot recover in simple substrate solutions such as phosphate buffer and the recovery process requires the synthesis of mucopeptide and ATP (Figs. 66 and 68). This indicates that the injury involves not only the lipo-polysaccharide layer of the cell wall but also the mucopeptide layer. It is well known that the cytoplasmic membrane lying immediately beneath the wall is so fragile that it has to be protected by the cell wall against osmotic forces. The wall must be rigid and there is abundant evidence that the mucopeptide component confers this property on the cell wall as a whole (Mandelstam and McQuillen, 1973). The cell wall mucopeptide of *S. heidelberg* probably undergoes some kind of change or degradation during cold storage in distilled water. This is further substantiated by the fact that injured cells died off much more rapidly than the normal cells in water at 20° (Fig. 69) and the recovering cells resumed their resistance to osmotic pressure gradually in accordance with the

increasing tolerance to desoxycholate (Fig. 71).

In conclusion, storage of *S. heidelberg* at refrigeration temperatures results in damage to the cell envelope of the organism and renders them sensitive to surface-active agents. The damage may be due to altered lipo-polysaccharide/or mucopeptide of the cell wall depending on the storage menstruum. The injury is reversible and is dependent on the pH and temperature and conceivably on enzymatic reaction(s). It is suggested that pre-enrichment procedures should be employed in the isolation and detection of salmonellae from foods stored at refrigeration temperatures, possibly in glucose-salt broth to compensate the phenomenon of 'minimal medium recovery'. The purpose of the pre-enrichment procedure would be to allow the cells to recover from the cold injury without being exposed to inhibitory or selective substances which might be unduly toxic to the injured cells. In addition, selective agents such as brilliant green agar that are not surface-active should be developed and employed. Since cold injury very often leads to increased permeability of the cell envelope, all plating diluents should be prepared in distilled water free of toxic trace elements. If direct enumeration of the organism in the food sample is required, appropriate inhibitors to prevent multiplication of the cells, but to allow recovery, could be added to the recovery menstruum.

The results reported in this thesis support earlier studies on the effects of environmental stress on injury to micro-organisms. Namely that injury is a very complex phenomenon and the extent of injury and recovery is markedly affected by experimental conditions. With increasing pressures for bacteriological standards for foods, it is

essential that these factors be recognized. Considering the different responses of different micro-organisms, it is clear that much more work in this field of study is essential.

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